Molecular Neurology, Research Programs Unit,
Faculty of Medicine, University of Helsinki

Doctoral Programme in Biomedicine, University of Helsinki

TISSUE-SPECIFIC IMPLICATIONS OF MITOCHONDRIAL DNA MAINTENANCE IN HEALTH AND DISEASE

Joni Nikkanen

ACADEMIC DISSERTATION

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Supervised by

Professor Anu Wartiovaara, MD, PhD,
Molecular Neurology, Research Programs Unit, University of Helsinki, Helsinki, Finland and Department of Neurology, Helsinki University Central Hospital, Helsinki, Finland

Reviewed by

Associate Professor Ville Hietakangas, PhD,
Department of Biosciences, University of Helsinki, Helsinki, Finland

Professor Laurence Bindoff, MD, PhD,
Department of Clinical Medicine (K1), University of Bergen, Bergen, Norway

Discussed by

Professor Aleksandra Trifunovic, PhD,
CECAD Research Center, University of Cologne, Cologne, Germany

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“If your mouth is open, you are not learning” – Buddha

ABSTRACT

Defects of mitochondrial DNA (mtDNA) replication underlie common metabolic disorders. Despite mtDNA is degraded and synthesised in all cells containing mitochondria, mtDNA replication stress typically causes generation of mtDNA deletions or depletion of mtDNA copy number in muscle and brain, which manifest as mitochondrial myopathy (MM) or neurodegeneration, respectively. MtDNA replication defects, however, do not affect highly proliferative tissues, such as blood or intestine, despite their reliance on robust mtDNA replication to sustain high rates of proliferation. The mechanisms behind the tissue-specific manifestations of mtDNA replication defects remain unknown.

In this thesis, we aimed to identify the metabolic response pathways for mtDNA replication stress caused by a dominant Twinkle mtDNA helicase (*TWNK*) mutation leading to adult-onset MM. We collected samples from MM patients and a MM mouse model, Deletor mouse, for a multi-tissue metabolic characterisation. The study revealed that MM induces a metabolic reprogramming in the muscle directing glucose consumption to serine synthesis to support cysteine production by transsulfuration pathway. The metabolic response also remodels vitamin B9 (folate) metabolism and causes imbalance in whole-cellular deoxynucleotide triphosphate (dNTP) pools, which are likely novel contributors in the progressive mtDNA mutagenesis. The characterised metabolic stress response for the mtDNA replication defect was found to be orchestrated by one master regulator, mechanistic target of rapamycin complex I (mTORC1). The mTORC1-mediated stress response appeared to promote disease progression, and an mTORC1 inhibitor, rapamycin, remarkably improved the mitochondrial muscle disease. It ameliorated the typical hallmarks of MM: the number of ragged red fibers (RRFs) and the amount of mtDNA deletions were reduced after rapamycin treatment.

In the second part of this thesis, we studied the transcription regulation of mtDNA replication machinery. We identified a complex regulatory locus for DNA polymerase gamma (*POLG*) by *in silico* predictions, which were verified *in vivo*. The regulatory non-coding locus drives *POLG* expression specifically in the sensory interneurons of the spinal cord and oculomotor nucleus, which we found to degenerate in POLG patients. The death of these neurons might be the underlying cause of sensory neuropathy and progressive external ophthalmoplegia (PEO), which are typical clinical findings in POLG disorders. The identified regulatory locus functions through a non-coding RNA and three enhancer elements but also contains MIR-9, which targets the mitochondrial folate pathway by downregulating methylene tetrahydrofolate dehydrogenase 2 (MTHFD2). Therefore, this locus provides an unexpected link between the expression of *POLG* and mitochondrial folate metabolism.

In conclusion, our work has identified novel contributors in the tissue-specific manifestations of mitochondrial diseases and offers multiple novel treatment targets for mitochondrial disorders, which currently lack effective treatment options.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Nikkanen J.**, Forsström S., Euro L., Paetau I., Kohnz R.A., Wang L., Chilov D., Viinamäki J., Roivainen A., Marjamäki P., Liljenbäck H., Ahola S., Buzkova J., Terzioglu M., Khan N.A., Pirnes-Karhu S., Paetau A., Lönnqvist T., Sajantila A., Isohanni P., Tyynismaa H., Nomura K., Battersby B.J., Velagapudi V., Carroll C.J., Suomalainen A. Mitochondrial DNA Replication Defects Disturb Cellular dNTP Pools and Remodel One-Carbon Metabolism. Cell Metabolism (2016) Apr 12;23(4):635-48.

II Khan N.A., **Nikkanen J.**, Yatsuga S., Jackson C., Wang L., Pradhan S., Kivelä R., Pessia A., Velagapudi V., Suomalainen A. mTORC1 Regulates Mitochondrial Stress Response and Mitochondrial Myopathy Progression. Cell Metabolism (2017) Aug 1;26(2):419-428.e5.

III **Nikkanen J.**, Landoni J.C., Balboa D., Haugas M., Partanen J., Paetau A., Isohanni P., Brilhante V., Suomalainen A. A Complex Enhancer-Non-Coding-RNA Locus Regulates Mitochondrial DNA Maintenance in Central Nervous System. *Submitted.*

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

[^{18}F]-FDG	2-[^{18}F]-fluoro-2-deoxy-D-glucose
1C	one-carbon
ad	autosomal dominant
ADP	adenosine diphosphate
ar	autosomal recessive
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary DNA
CNS	central nervous system
CoA	coenzyme A
COX	cytochrome c oxidase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DHS	DNase I hypersensitive site
dNTP	deoxynucleotide triphosphate
dRP	deoxyribose phosphate
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
dUrd	deoxyuridine
EE	enhancer element
EEL	Enhancer element locator
ENCODE	encyclopedia of DNA elements
EOM	extraocular muscle
ES cells	embryonic stem cells
fMet	N-formylmethionine
GTP	guanosine triphosphate
IOSCA	infantile-onset spinocerebellar ataxia syndrome
kb	kilo base pair
lncRNA	long non-coding RNA
MDS	mtDNA depletion syndrome
MIRAS	mitochondrial recessive ataxia syndrome
MM	mitochondrial myopathy
MNGIE	mitochondrial neurogastrointestinal encephalopathy
mRNA	messenger RNA
MS	mass spectrometry
mtDNA	mitochondrial DNA
mTORC1	mechanistic (mammalian) target of rapamycin
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate

NEO	neomycin
NR	nicotinamide riboside
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEO	progressive external ophthalmoplegia
ROS	reactive oxygen species
RRF	ragged-red fiber
rRNA	ribosomal RNA
SAH	s-adenosyl-homocysteine
SAM	s-adenosyl-methionine
SDH	succinate dehydrogenase
TCA cycle	tricarboxylic acid cycle
TF	transcription factor
TFBS	transcription factor binding site
Thd	thymidine
THF	tetrahydrofolate
tRNA	transfer RNA
UPLC	ultra performance liquid chromatography
UTR	untranslated region
WT	wild type

1 INTRODUCTION

Cellular metabolism is compartmentalised: major metabolic pathways function in organelles that are separated from the cytoplasm by lipid membranes. The isolated nature of cellular organelles enables them to execute processes that require specialised environment. For example, low pH is essential for the function of lysosomal enzymes, whereas highly regulated redox environment is required for mitochondrial oxidative phosphorylation (Hu et al., 2008). Mitochondria also perform a variety of other essential metabolic functions and are, therefore, critical for cellular metabolism. Some pathways function solely within the mitochondrial compartment (e.g. the tricarboxylic acid (TCA) cycle), but others function partly in cytoplasm and partly in mitochondria (e.g. the folate cycle). Cellular metabolism is, therefore, highly dependent on the efficient cross-talk of the cytoplasmic and mitochondrial compartments, but the signalling between these compartments is currently poorly characterised. Also, the cytoplasmic responses to mitochondrial dysfunction remain largely unknown.

MtDNA replication defects are a major cause of mitochondrial disorders. Mutations in mtDNA replication enzymes cause the loss of mtDNA integrity and induce mtDNA depletion or accumulation of mtDNA deletions, which underlie various human disorders. The loss of mtDNA integrity has detrimental consequences on cellular metabolism beyond the respiratory chain function and adenosine triphosphate (ATP) production since it has also been linked to mitochondrial translation defects, which induce mitochondrial membrane stress. Functional mitochondrial translation is critical in maintaining mitochondrial membrane potential, which is required for efficient mitochondrial membrane trafficking.

Mitochondrial diseases belong to the group of metabolic disorders, but the exact biochemical consequences of mtDNA replication defects to metabolic pathways are not well known. Identifying the molecular consequences of mitochondrial dysfunction to cellular metabolism has a critical role in designing effective interventions and developing new treatments. Currently no cure can be offered to patients suffering from mitochondrial diseases, but the available treatments only alleviate disease symptoms and provide palliative care.

In this thesis, we describe the metabolic consequences of mtDNA replication defects *in vivo*.

2 REVIEW OF THE LITERATURE

2.1 THE STRUCTURE AND FUNCTIONS OF MITOCHONDRIA

2.1.1 THE ORIGIN OF MITOCHONDRIA

Mitochondria are intracellular organelles of eukaryotic cells. They form a dynamic network, which undergoes constant fission and fusion events (Figure 1). Mitochondria are thought to originate from an endosymbiotic event where an ancestral prokaryote, α-proteobacterium, was engulfed by another cell (Margulis, 1970). The current structure of mitochondria resembles the ancestral prokaryote as both have a double-membrane structure, circular genome and their own transcription and translation systems. Prokaryotes and mitochondria both transcribe a long polycistronic RNA product and use N-formylmethionine (fMet) in translation initiation (Gray, 2012). Also, some mitochondrial proteins, such as 60- and 70 kDa heat shock proteins (HSP60, HSP70), have α-proteobacterium homologues, but they are currently encoded by the ancestral host genome, translated in the cytoplasmic ribosomes and imported to mitochondria, where they originally functioned. This phenomenon is called endosymbiotic gene transfer, and it happened during the reduction of mtDNA molecule. (Timmis et al., 2004)

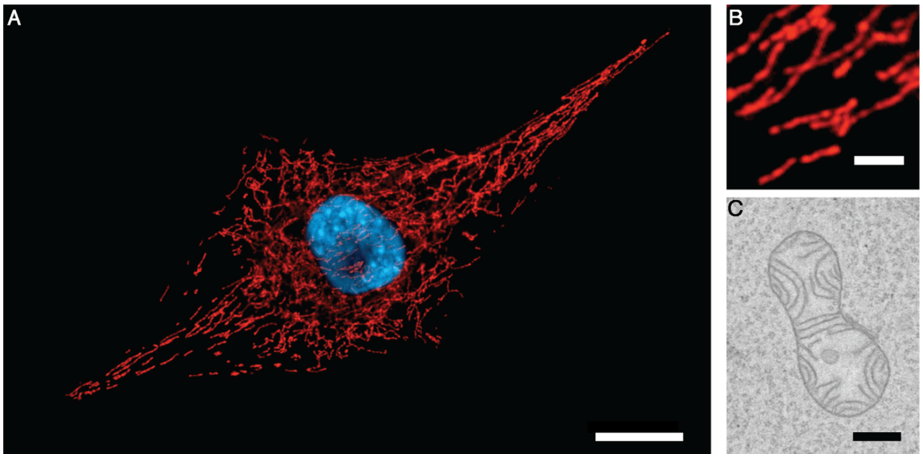

Figure 1 (A) Human myoblast showing mitochondrial network in red (Mitotracker) and DNA in blue (DAPI). Scale bar: 10 µm. (B) Magnification of mitochondria from (A). Scale bar: 10 µm (C) Electron micrograph of a mitochondrion from human fibroblast. Scale bar: 200 nm. Imaging by Christopher Jackson.

Whereas α-proteobacterium is generally accepted to be the ancestor of mitochondria, different theories exist for the nature of the host and the beneficial function of the cytoplasmic parasite for the host cell. The most common view states that the host cell was an anaerobic eukaryote, which engulfed the mitochondrion by active phagocytosis. The initial benefit of the mitochondrial endosymbiont for the host cell might have been its ability to consume and, thereby, detoxify oxygen from the environment. This theory assumes that all initial eukaryotes, except the ones with mitochondria, went extinct as no eukaryotic cells without mitochondria exist. Another theory suggests that the original host cell was an archaea that benefited from the endosymbiont's ability to produce H_2, which the host utilised as a source of energy and electrons (Embley and Martin, 2006).

2.1.2 THE FUNCTIONS OF MITOCHONDRIA

Mitochondria perform various anabolic and catabolic activities. Most mitochondrial functions, however, do not have an essential role for eukaryotic life. For example, the complete loss of mtDNA molecule, which encodes essential subunits for cellular respiration, does not show detrimental consequences in yeast *Saccharomyces cerevisiae*, which is able to grow without mtDNA in the presence of a fermentable carbon source (Williamson, 2002).

The most conserved essential mitochondrial function, present in all eukaryotes, is the synthesis of iron-sulphur clusters, which happens in the mitochondrial matrix (Lill and Kispal, 2000). Mutant yeast strains incapable of synthesising iron-sulphur clusters fail to grow under any conditions due to the lack of functional extra-mitochondrial iron-sulphur cluster proteins (Kispal et al., 2005; Lill et al., 1999). However, most mitochondrial functions, such as beta-oxidation and the function of the respiratory chain, are essential for higher organisms, such as humans, and defects in those pathways underlie a broad spectrum of human diseases (Gerards et al., 2016; Wanders et al., 1989).

2.1.2.1 Respiratory chain and oxidative phosphorylation

The best-known role of mitochondria is to provide ATP molecules through oxidative phosphorylation (OXPHOS) (Mitchell, 1961). This is catalysed by five large enzyme complexes, which reside in the folded inner membrane of the mitochondria (Figure 2). The respiratory chain consists of complexes I-IV that pump protons to create an electrochemical gradient across the inner mitochondrial membrane, which is utilised by complex V to convert adenosine diphosphate (ADP) to ATP (Boyer et al., 1973). Most of the subunits of the five complexes are encoded in the nucleus and imported into mitochondria, but 13 of the hydrophobic core subunits of complexes I, III, IV and V are encoded by

mtDNA and translated by mitochondrial ribosomes. The successful assembly of the complexes requires an exceptional coordination of mitochondrial protein import, expression of the mitochondrial genome and insertion of the subunits into the inner membrane.

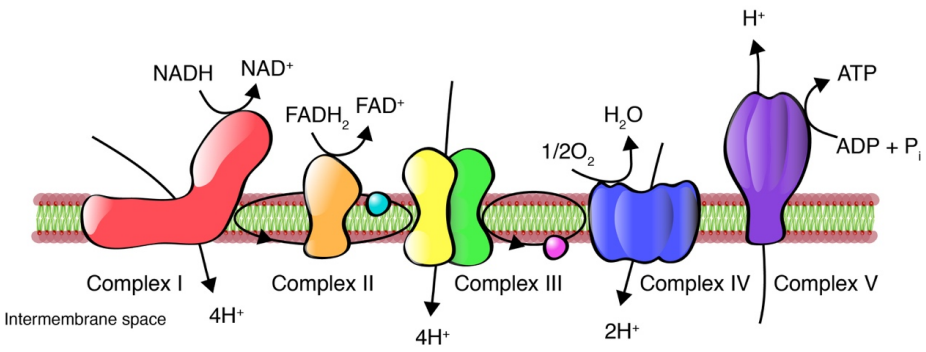

Figure 2 Schematic picture of oxidative phosphorylation. Electron carriers ubiquinone (blue ball) and cytochrome c (purple ball) shuttle electrons to respiratory chain complexes. The complexes pump protons to the intermembrane space to create an electrochemical gradient across the inner mitochondrial membrane. This gradient is utilised by complex V to convert ADP and Pi to ATP.

Complex I, or NADH dehydrogenase, consists of 45 protein subunits, 7 of which are mitochondrial encoded. This complex is one of the two entry points of the respiratory chain as it obtains two electrons by oxidising two NADH molecules that derive from the TCA cycle. The electrons are shuttled through iron-sulphur clusters to a lipid-soluble carrier, ubiquinone, which is converted to its reduced form, ubiquinol (Baradaran et al., 2013). During this process four protons are pumped to the intermembrane space. Importantly, in addition to the reduction of ubiquinone and proton pumping, the role of complex I in NADH oxidation is critical in providing sufficient NAD^+ flux to the TCA cycle (Martinez-Reyes et al., 2016). An additional link between the TCA cycle and the respiratory chain is complex II, succinate dehydrogenase, which is the only complex that is part of both of these pathways. Complex II is the second entry point to the respiratory chain, but instead of NADH, it uses flavin adenine dinucleotide ($FADH_2$) in the reaction that does not involve proton pumping but reduces one molecule of ubiquinone and converts succinate to fumarate.

The ubiquinol molecules provided by complexes I and II diffuse within the inner mitochondrial membrane and are used by complex III, coenzyme Q:cytochrome c oxidoreductase. This complex, as its name suggests, uses ubiquinol to reduce two molecules of cytochrome c, which is the other electron carrier in the respiratory chain. As a result of this reaction, complex III pumps

two protons to the intermembrane space. The reduced cytochrome c is utilised by complex IV, cytochrome c oxidase, which is the last enzyme complex of the respiratory chain. It transfers the electrons from cytochrome c to O_2 producing H_2O, and protons are pumped across the membrane during this process. (Saraste, 1999)

The energy stored in the electrochemical gradient, created by the respiratory chain, is utilised by complex V, ATP synthase, which converts ADP and P_i into ATP (Mitchell, 1961). ATP synthase consists of a water-soluble F_1 part and a F_0 ring-like structure, which is located in the membrane (Abrahams et al., 1994). These two parts are connected by the central and peripheral stalks. When the protons flow through the F_0 ring structure, the membrane-bound F_0-part and the central stalk rotate, but F_1-part is prevented from rotating by the peripheral stalk. The rotation of the central stalk within the F_1-part activates the reaction of the ATP synthesis (Abrahams et al., 1994; Gresser et al., 1982; Stock et al., 2000).

The OXPHOS complexes form so called supercomplexes where multiple complexes are physically and functionally linked (Schagger and Pfeiffer, 2000). They are found in multiple respiratory chain complex combinations: (I+III), (I+III+IV) and (III+IV), but complex II has not been found to associate with these supercomplexes (Dudkina et al., 2005; Heinemeyer et al., 2007; Schagger and Pfeiffer, 2000). Supercomplexes are characterised from multiple species and are detected by blue-native polyacrylamide gel electrophoresis and electron microscopy single-particle analysis (Chaban et al., 2014; Minauro-Sanmiguel et al., 2005). Also, ATP synthase is found as a dimeric complex in yeast, Arabidopsis, Bos taurus and several other species (Arnold et al., 1998; Eubel et al., 2003). The dimerisation of ATP synthase has been associated to crista formation and to maintenance of the folded nature of the mitochondrial inner membrane (Dudkina et al., 2010; Strauss et al., 2008). This is supported by studies in yeast, where disintegration of the ATP synthase dimer disrupts the mitochondrial ultrastructure leading to onion-like and balloon-shaped mitochondria (Davies et al., 2012).

2.1.2.2 One-carbon metabolism

One-carbon (1C) metabolism is a metabolic pathway, which is essential to virtually all cell types and all organisms. It provides 1C-units to major anabolic cellular pathways: nucleotide synthesis, methylation reactions and mitochondrial translation initiation (Figure 3) (Tong et al., 2009). The carrier of activated 1C-units is folate (vitamin B9), which functions in its reduced form, tetrahydrofolate (THF), and accepts 1C-units from 1C-donors, mainly serine and glycine (Shane and Stokstad, 1985). THF can function in several different oxidative states, enabling it to serve as an enzyme cofactor in different reactions. The conversion of different THF intermediates forms a cyclic pathway, which has cytoplasmic and mitochondrial parts. The mitochondrial compartment is thought to have its own stable THF

intermediate pool, maintained by active transport of THF into mitochondria by the function of SLC25A32. No exchange of activated THF forms between the two compartments have been reported. (Cook and Blair, 1979; Titus and Moran, 2000; Zamierowski and Wagner, 1977)

Mitochondrial folate metabolism uses THF and 1C-donors serine, glycine or sarcosine as inputs and produces fMet for mitochondrial translation initiation, NADPH and formate (Fan et al., 2014). Formate can pass through mitochondrial membrane without active transport, and it is suggested to be the major 1C-source for cytoplasmic 10-formyl-THF used for inosine monophosphate production, which is the end product of *de novo* purine synthesis. 10-formyl-THF is used by GAR transformylase and AICAR transformylase, which catalyse two steps in the pathway. Compromised 10-formyl-THF production affects first AICAR transformylase given its lower K_m for 10-formyl-THF compared to GAR transformylase (Mueller and Benkovic, 1981; Smith et al., 1981). Suboptimal function of AICAR transformylase leads to accumulation of its substrate, AICAR, which is a direct activator of AMP-activated protein kinase (AMPK) (Herbert et al., 1964; Li et al., 2016). AMPK plays a critical role in cellular energy homeostasis and when activated, blocks cellular anabolism (Hardie et al., 2012). The availability of 10-formyl-THF, thereby, links nutrient (folate) availability and mitochondrial folate metabolism to AMPK-mediated nutrient sensing.

To date, NADPH production by folate cycle has only been observed in cultured cells and its *in vivo* significance remains unclear. *In vitro* data indicate that NADPH from the folate cycle is critical in reduction of oxidised glutathione and, thereby, important for reactive oxygen species (ROS) scavenging and the function of the hydrogen donor system (Fan et al., 2014).

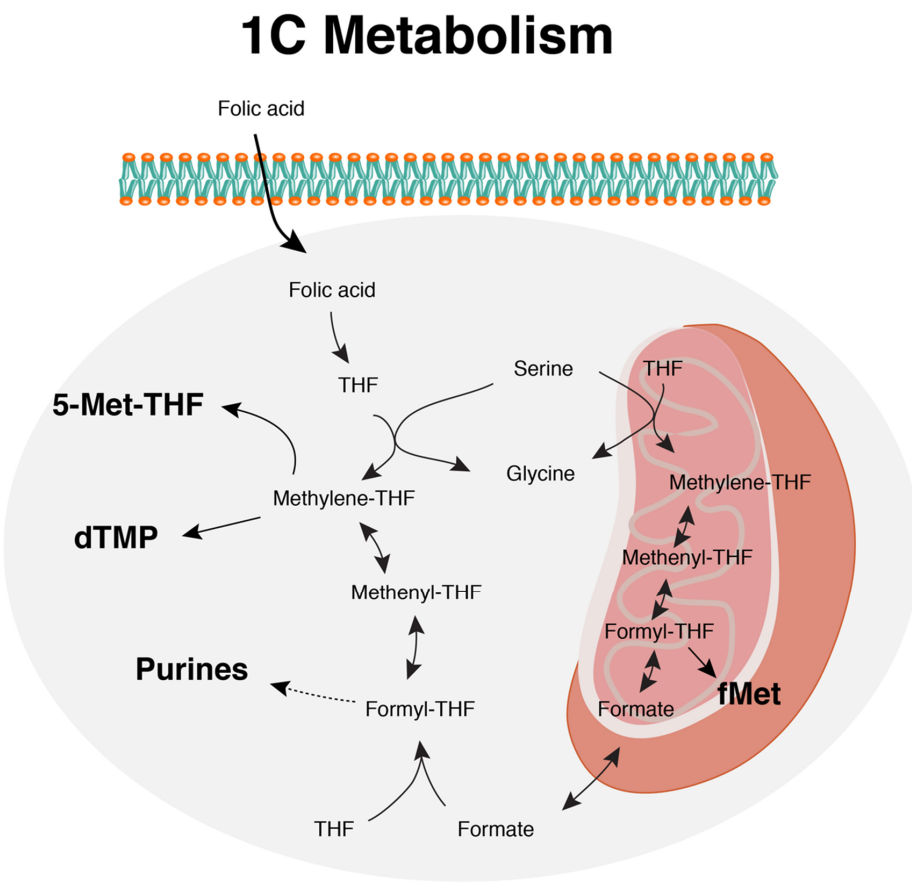

Figure 3 Schematic picture of 1C-metabolism and folate intermediates. End-products shown in bold.

In addition to purine synthesis, cytoplasmic folate pathway is critical for maintaining the function of the methyl cycle, which provides s-adenosyl-methionine (SAM) (Figure 4). SAM is the sole methyl donor in the cell and, therefore, the substrate for all methyltransferases, which catalyse the methylation of DNA, RNA, proteins and metabolites (Anderson et al., 2012; Zatz et al., 1981). The synthesis of SAM is dependent on the conversion of homocysteine to methionine. This is catalysed by methionine synthase, which uses 5-methyl-THF as a cofactor (Stipanuk, 2004). When the intake of dietary folate is compromised and intracellular folate intermediate pools are reduced, homocysteine accumulates as one of the first signs of folate deficiency (Collaboration, 1998). Increased level of homocysteine has been associated to several human conditions, e.g. cardiovascular diseases, highlighting the importance of sufficient folate intake. In addition, accumulation of homocysteine can also bind to s-adenosyl-homocysteine hydrolase (SAHH), which functions as the scavenger of the side product of methylation reactions,

s-adenosyl-homocysteine (SAH) (Palmer and Abeles, 1979). The binding of homocysteine to SAHH inhibits its activity resulting in accumulation of intracellular SAH, which is a potent inhibitor of methyltransferases and, thereby, disturbs different methylation reactions in the cell (Hoffman et al., 1980; James et al., 2002).

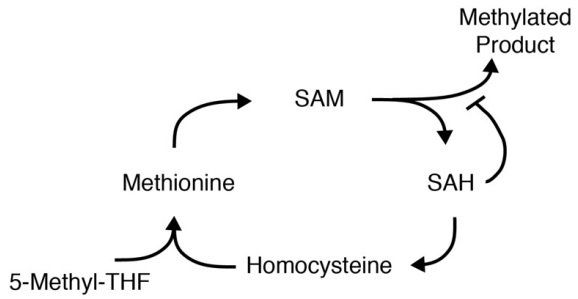

Figure 4 Schematic picture of methyl cycle. Abbreviations: SAM, S-adenosyl-methionine; SAH, S-adenosyl-homocysteine; THF, tetrahydrofolate

2.1.3 MITOCHONDRIAL DNA AND ITS REPLICATION

Mitochondria harbour their own genome, which is 16 569 base pairs (bp) long and encodes 13 polypeptides, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (Anderson et al., 1981). The products of all genes encoded by mtDNA are used within the organelle: protein-coding genes encode subunits of OXPHOS and the functional RNAs are required for mitochondrial translation (Figure 5). The vast majority of the mitochondrial proteome (~1200 proteins in total) is encoded by the nuclear DNA, translated in cytoplasmic ribosomes and imported into mitochondria (Calvo et al., 2016). The imported proteins have their specified functions and are responsible e.g. for the replication of mtDNA, which takes place in all tissues, including post-mitotic tissues in which nuclear DNA is not replicating. The number of mtDNA molecules per cell varies from hundreds to thousands of copies depending on the cell type. This creates, together with the rate of cell division and mtDNA turnover, a cell-specific pressure on the mtDNA replication machinery. (Chan et al., 2013; Miller et al., 2003). The minimal mtDNA replisome *in vitro* includes POLG, TWNK and SSBP1, but several other enzymes are involved in mtDNA replication *in vivo* (Korhonen et al., 2004). These include enzymes for dNTP synthesis, topoisomerase to relieve torsional stress and RNA polymerase to synthesise RNA primers for replication initiation (Fuste et al., 2010).

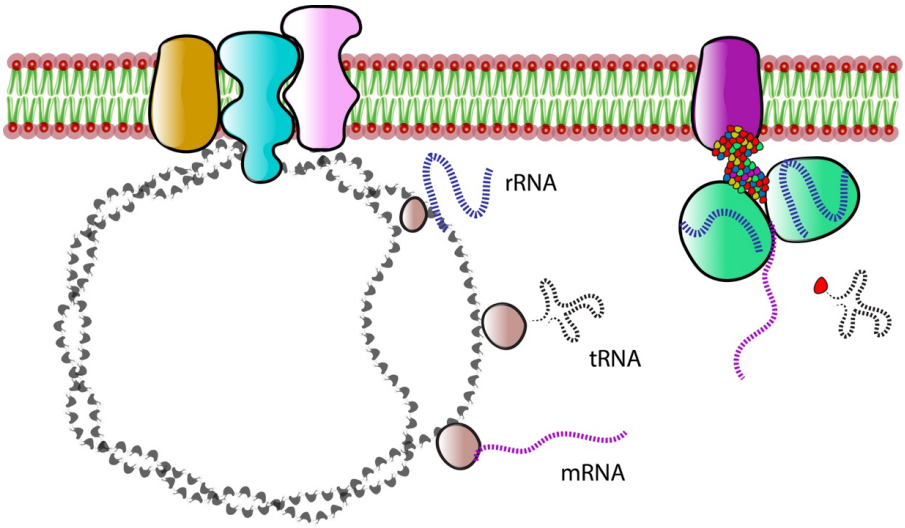

Figure 5 Mitochondrial DNA and mitochondrial translation machinery. MtDNA encodes subunits of the OXPHOS, tRNAs and rRNAs, which are transcribed as a polycistronic RNA product and processed to give rise to different RNA species. The functional RNAs are used in the translation of subunits of the OXPHOS complexes by mitochondrial ribosomes (green balls). The translation is coupled with insertion of the synthesised protein into a functional complex.

The current understanding of mtDNA replication is not complete, and different replication models have been proposed. In the strand-displacement model, replication initiates in the D-loop and continues until the origin of the light strand replication is reached, which initiates the replication of the light strand to the opposite direction (Clayton, 1982). This was initially proposed as the model for mtDNA replication due to detection of single-stranded replication intermediates in electron micrographs (Clayton, 1982; Robberson et al., 1972). An alternative model suggests that mtDNA is replicated through a conventional strand-coupled mechanism. This is supported by detection of double-stranded mtDNA replication intermediates, which can be separated by two-dimensional agarose gel electrophoresis (Holt et al., 2000). Also, detection of multiple lagging strand initiation sites further supports this model of mtDNA synthesis (Brown et al., 2005). Later discoveries revealed that the lagging strand can be synthesised first as RNA, which is subsequently matured into DNA (Yasukawa et al., 2006).

2.2 MITOCHONDRIAL DNA MAINTENANCE ENZYMES

2.2.1 MITOCHONDRIAL TRANSCRIPTION FACTOR A

Nuclear DNA is compactly packed into nucleosomes, which are histone complexes wrapped with DNA. Similar nucleosome-like structures exist in mitochondria since the ~5 μm long mtDNA cannot exist as a free molecule within a typical mitochondrion (Kukat and Larsson, 2013; Nass, 1969). These structures containing proteins and DNA are called nucleoids, and they are likely to be attached to the inner mitochondrial membrane. A critical protein for the packaging of mtDNA into nucleoids is mitochondrial transcription factor A (TFAM), which can bind, bend and unwind mtDNA without sequence specificity (Fisher et al., 1992). One nucleoid is estimated to have only one copy of mtDNA, but the complete protein composition of the mitochondrial nucleoid is still under investigation (Kukat et al., 2011). In addition to TFAM, nucleoids consist of enzymes of the mitochondrial replication and transcription machineries (Bogenhagen et al., 2008; Bogenhagen et al., 2003). TFAM is an essential protein in mtDNA maintenance since knocking it out from mouse leads to loss of mtDNA copy number and embryonic lethality (Larsson et al., 1998). Overexpressing TFAM in mouse increases mtDNA copy number indicating that TFAM regulates mtDNA copy number *in vivo* (Ekstrand et al., 2004).

TFAM also functions as a transcription factor (TF), and it is an indispensable factor in the expression of mtDNA (Fisher and Clayton, 1988; Shi et al., 2012). The mitochondrial transcription machinery has been reconstituted *in vitro* and has three components: mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor B2 and TFAM (Falkenberg et al., 2002). Also, mtDNA replication *in vivo* is dependent on TFAM as the synthesis of RNA primers is catalysed by the transcription machinery (Fuste et al., 2010).

2.2.2 DNA POLYMERASE GAMMA

DNA polymerases replicate DNA for transmission of genetic material to daughter cells. The replication of nuclear DNA is mediated by several different DNA polymerases, but DNA polymerase gamma (POLG) is thought to be the main mitochondrial DNA polymerase (Bolden et al., 1977). POLG is a heterotrimeric enzyme consisting of one catalytic subunit (POLG) and two accessory subunits (POLG2), which regulate the processivity of the enzyme complex (Yakubovskaya et al., 2006). Abolished POLG expression in mouse is embryonically lethal due to depletion of mtDNA copy number between embryonic days E7.5-E8.5 suggesting that no other polymerase has redundant mtDNA replicative activity during embryogenesis (Hance et al., 2005).

The catalytic subunit, POLG, consists of three main domains: polymerase domain, exonuclease domain and the spacer region, which connects the two

catalytic domains. The crystal structure of POLG revealed that the spacer region is critical in binding the two accessory subunits (Lee et al., 2009). This feature provides an exceptionally high polymerase processivity compared to the other members of the Pol I family, which lack the spacer region (Lee et al., 2009). POLG also has exceptional fidelity due to intrinsic 3'-5' exonuclease and 5'-deoxyribose phosphate (dRP) lyase activities (Lim et al., 1999; Longley et al., 1998a; Longley et al., 1998b). Yeast, *Drosophila* and mouse mutants lacking POLG proof-reading activity accumulate mtDNA point mutations and show signs of premature ageing (Bratic et al., 2015; Foury and Vanderstraeten, 1992; Trifunovic et al., 2004).

The role of mtDNA repair has not been vastly investigated to date, and therefore, its significance in mitochondrial function and disease is still not fully understood. The best characterised mode of repair is base excision repair, which has been suggested to be the main repair pathway in mitochondria (Kazak et al., 2012). The recognition and cleavage of a damaged base is catalysed by DNA glycosylases, e.g. UNG1 and MYH, which are targeted to mitochondria (Nakabeppu, 2001; Nilsen et al., 1997; Slupphaug et al., 1993). Monofunctional DNA glycosylases leave a 5'-terminal dRP sugar in the cleaved site, which needs to be removed before gap filling. This is catalysed by POLG dRP-lyase activity (Longley et al., 1998a). After processing, the gap is filled by POLG, and the damage is repaired preventing a point mutation from occurring.

2.2.3 TWINKLE HELICASE

Mitochondrial helicase Twinkle was found in 2001 when Spelbrink et al. reported mitochondrial localisation of a phage T7 gene 4-like primase/helicase encoded by *TWNK* (Spelbrink et al., 2001). TWNK forms hexameric ring structures and unwinds double-stranded DNA, but despite the homology between phage T7 primase/helicase, TWNK has lost its primase function in mammals (Korhonen et al., 2003; Shutt and Gray, 2006). The RNA primers for initiation of mtDNA synthesis in mammals are synthesised by mitochondrial RNA polymerase (POLRMT) (Fuste et al., 2010; Wanrooij et al., 2008).

The evidence suggests that TWNK works as a licensing factor in mtDNA replication initiation since the amount of TWNK highly correlates with mtDNA copy number, and the overexpression of TWNK increases mtDNA copy number in muscle, heart and brain in mice (Tyynismaa et al., 2004; Ylikallio et al., 2010). This is further supported by a study which reports induced mtDNA replication and enlarged nucleoids in mice overexpressing TWNK (Ylikallio et al., 2010).

2.3 GENOMIC REGULATION OF MTDNA MAINTENANCE PROTEINS

2.3.1 REGULATORY ELEMENTS OF NUCLEAR DNA

The control of gene expression is a complex interplay between transcriptional and post-transcriptional regulatory mechanisms forming a unique regulatory network to control the expression of each gene. There are circa 20,000 genes in the human genome, but only a limited number of them is expressed simultaneously (Collins et al., 2003). The set of expressed genes in a cell defines its structure and functions forming specialised cell types, or more broadly, different tissues. Some genes work as housekeeping genes and are required for the basic metabolism of the cell and, therefore, need to be continuously expressed. On the other hand, the expression of some genes is restricted to a specific tissue at a certain time point requiring strict temporal and spatial control (Goldhamer et al., 1995). These highly specific requirements for gene expression are achieved by multilevel regulatory stages.

Recent advances in DNA sequencing methods have provided an excellent base for comparative genomics and have also allowed methodological development for genome-wide analyses of the chromatin state across different species and tissues. Genome-wide approaches were used in the encyclopedia of DNA elements (ENCODE) project, which in consortium performed several state-of-the-art experiments with a large set of tissues and cell lines to characterise genomic DNA elements and chromatin structure (Consortium, 2012). This work included genome-wide analysis of transcription factor binding sites (TFBSs), DNA methylation profiles, DNase I hypersensitive sites (DHSs) and RNA sequencing (Consortium, 2012). All ENCODE data sets are available online for the research community and were also utilised in this thesis.

2.3.1.1 Proximal promoter

The initiation of transcription takes place in the promoter, which provides a docking site for the transcription machinery in the immediate upstream region of the transcription start site. Mammalian promoters contain various conserved sequence elements, which are recognised and bound by TFs. The TFs in the proximal promoters can induce or repress gene expression or provide binding sites for distal regulatory elements functioning *in cis* usually leading to more fine-tuned gene regulation, e.g. tissue-specificity of transcription (Carey and Smale, 2000; Perkins et al., 1996).

2.3.1.2 Distant-acting regulatory elements

The recruitment of RNA polymerase to proximal promoter and its release to initiate transcription is often regulated by distal DNA sequences, which are called enhancer elements. These elements are DNA sequences that are recognised and bound by TFs, which form DNA-protein interactions. The enhancer-bound TFs can in unison activate gene transcription independent of the orientation, distance or location of the enhancer element in respect to the target gene (Banerji et al., 1981). Some enhancers are even known to regulate genes located in other chromosomes (Geyer et al., 1990).

The exact mechanisms how enhancer elements signal to the target promoter, are still under investigation. Recent development in microscopy and the discovery of chromosome conformation capture have revealed that an enhancer element and the target promoter are commonly brought to close proximity by DNA looping (Figure 6) (Dekker et al., 2002). These interactions are, however, transient and the dynamic nature of chromatin state poses challenges in interpreting data regarding the mechanisms of gene regulation. In some cases, the looping of DNA for enhancer-promoter interaction is achieved by the function of the mediator complex, which is a co-activator complex consisting of approximately 30 subunits (Kagey et al., 2010). It signals between the enhancer and transcription machinery via the interaction with site-specific factors and RNA polymerase of the proximal promoter (Kagey et al., 2010; Malik and Roeder, 2010). Alternative modes of action are also reported to mediate enhancer function. For example, the phosphorylation of serine at amino acid position 10 of histone H3 in an enhancer triggers a cascade where an adaptor protein, 14-3-3, binds the phosphorylated histone and recruits a histone acetyltransferase. The histone acetyltransferase can further modify the epigenetic landscape of the surrounding genomic locus activating the transcription of the target gene, *FOSL1* (Figure 6) (Zippo et al., 2009).

Genome-wide studies have characterised the landscape of the genomic regulatory elements in detail identifying the regulatory regions of more than 100 different cell and tissue types (Consortium, 2012). However, these studies were mostly conducted in cell lines or by using whole-tissue homogenates and identified relatively general regulators and did not find elements that drive expression in highly restricted cell populations. Therefore, *in silico* predictions of regulatory elements and their validation *in vivo* is needed to identify elements with highly spatially restricted function (Hallikas et al., 2006).

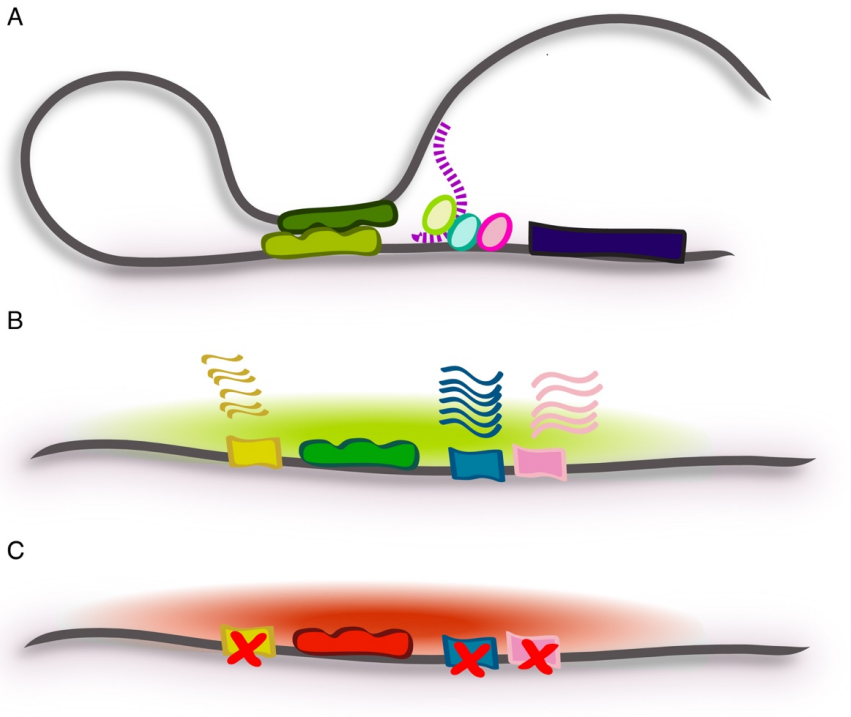

Figure 6 Gene regulation mechanisms by lncRNAs and enhancer elements. (A) Enhancer elements create DNA loops to bring chromatin structures to close proximity. LncRNA (purple dashed line) acts as a protein scaffold to recruit transcription machinery to the proximal promoter to initiate RNA synthesis. (B) Enhancers can modify the epigenetic landscape of chromatin regions to active or (C) repress gene expression.

2.3.2 LONG NON-CODING RNAS REGULATE GENE EXPRESSION

Long non-coding RNAs (lncRNAs) are generally defined as > 200 bp long RNA transcripts that do not encode proteins but regulate the expression of other genes (Rinn et al., 2007; Rutenberg-Schoenberg et al., 2016). LncRNAs are, similarly to protein coding genes, transcribed by Pol II, spliced, capped and polyadenylated but are not translated into protein and perform active functions as RNA molecules. The classification of lncRNAs, however, is problematic since the general definition of lncRNAs is negative, i.e. the lack of protein coding property is used as an inclusion criterion. However, long RNA transcripts are likely to have, even by chance, stretches that have start and stop codons and can theoretically encode proteins that are > 100 amino acids long. Also, some RNAs can act as coding transcripts and have dual roles as regulatory RNAs the functions of which are independent of translation (Hube et al., 2006). To evaluate the potential of an RNA transcript to encode a protein, different comparative and experimental approaches can be used. In

contrast to genes encoding functional RNAs, protein coding genes often have a strong selection against variants that change the protein sequence (missense or frameshift variants), and they often contain conserved structural domains (Yang and Bielawski, 2000). In addition, a recently developed method to study RNA molecules that are bound by ribosomes (ribosomal footprint profiling) and are, therefore, translated into proteins, is useful for experimentally identifying lncRNAs by revealing protein-coding RNA transcripts (Guttman et al., 2013).

The functional roles of lncRNA are diverse, and new mechanistic insight into biochemical activities of lncRNAs are constantly discovered (Tay et al., 2014). In general, lncRNAs (1) act locally at the sites they are transcribed or (2) function *in trans* and regulate chromatin state at sites that are independent of the transcription site of the lncRNA. However, this classification is not exhaustive and many lncRNAs fall in between these classifications (Dimitrova et al., 2014; Huarte et al., 2010).

The flagship example in the field of lncRNAs is the X chromosome inactivation by *Xist*. In females, the expression of one of the two X chromosomes is repressed balancing the expression of the chromosome in females and males. The search for the factor mediating this inactivation led to the discovery of a large transcript (~18 kb in mice) that was first considered to be a protein-coding mRNA but eventually was found to be a functional RNA and the master regulator of X chromosome inactivation (Gendrel and Heard, 2014; Lee, 2009). The *Xist* transcript spreads along one of the X chromosomes coating the majority of the chromosome and eventually leading to major chromatin remodelling, which includes histone modifications and DNA methylation leading to silencing of the whole chromosome (Plath et al., 2003).

As mentioned above, lncRNAs can also act locally near the site of their transcription and assemble with protein complexes (Mao et al., 2011). These protein complexes are often chromatin modifying proteins that activate or repress gene expression (Brockdorff, 2013). One early example of locally acting lncRNAs is *Airn*, which was originally thought to suppress the expression of the antisense gene *Igf2r* by recruiting chromatin modifying factors (Wutz et al., 1997). Functional studies, however, indicated that the RNA sequence of *Airn* was not critical for silencing of *Igf2r*, but the act of its transcription as an antisense strand was found to drive the suppression of the target gene (Latos et al., 2012). This example showed that the sequence of lncRNAs can be dispensable and underscores the need of critical evaluation of the biochemical properties and functional mechanisms of lncRNAs.

2.3.3 REGULATORY REGIONS AND DISEASE

Recent advances in high-throughput sequencing have led to the discoveries of thousands of genetic variants that modify common diseases, such as diabetes and Alzheimer's disease, and normal human phenotypes, e.g. circulating metabolite levels (Billings and Florez, 2010; Kettunen et al., 2016; Lambert et

al., 2013). Many of these variants are located outside the protein-coding regions indicating that they might affect gene expression. Also, mutations disturbing gene regulation are known to underlie many human disorders, and a few examples are presented in this chapter.

Mutations in enhancer regions lead to reduced expression of the target genes in specific cells causing highly tissue-specific manifestations (Smith and Shilatifard, 2014). One example of such case is a form of beta-thalassemia, which is a disease caused by insufficient synthesis of beta-chains of haemoglobin resulting in severe anaemia (Williams and Weatherall, 2012). A subset of beta-thalassemia patients was found to carry a large deletion upstream of the beta-globin gene, *HBB*, which did not affect its coding region (Kioussis et al., 1983; Van der Ploeg et al., 1980). DNase I hypersensitive experiments and reporter studies revealed this region to be a complex regulatory region, which drives the expression of *HBB* for haematopoiesis.

Also, translocation of a gene to the vicinity of a strong enhancer region can have detrimental consequences since the expression of the gene will be ectopically activated or increased in cells that normally do not express the gene or express it at low level. In Burkitt's lymphoma *MYC* is translocated to the chromosomal region where immunoglobulin is encoded (Haluska et al., 1986). The enhancer element in the region activates *MYC* expression, and since *MYC* is an oncogenic TF, the patients develop B cell lymphomas. Transgenic mice carrying *Myc* linked to the immunoglobulin enhancer also develop B cell malignancies indicating the causal role of the *MYC* translocation for the cancer in Burkitt's lymphoma patients (Adams et al., 1985).

Mutations in proteins which regulate or mediate the enhancer function, can also disturb gene regulation leading to human pathologies. Patients with Cornelia de Lange syndrome carry mutations in *SMC1*, *SMC3* or *NIPBL*, which all are subunits of the cohesin complex that has a role in the enhancer-promoter communication but also functions in chromosome segregation. This dual role of the cohesin complex raises the question, which of the functions is affected in the patient? Mutations in *NIPBL* are commonly found in the 5' untranslated region (UTR) affecting the expression of the gene (Borck et al., 2006). The human symptoms develop when *NIPBL* transcript level is reduced to 85%, but the chromosome segregation can still occur when only 13% of WT *NIPBL* is present. This indicates that Cornelia de Lange syndrome is likely to be caused due to defective enhancer-promoter communication (Dorsett and Krantz, 2009; Liu and Krantz, 2008).

2.3.4 REGULATORS OF NUCLEAR ENCODED MITOCHONDRIAL PROTEINS

The structure and number of mitochondria vary in different tissues depending on the metabolic and energetic needs of the tissue. The mitochondrial network is also highly dynamic, adapts to cellular requirements and responds to external stimuli by changing mitochondrial mass, morphology and gene

expression. Known inducers of mitochondrial function and biogenesis are, for example, stem cell differentiation and different physiological stimuli, e.g. exercise and thermogenesis in muscle and brown adipose tissue, respectively (Cuezva et al., 1997; Enriquez et al., 1999; Hood, 2001; Klingenspor et al., 1996). Furthermore, mitochondrial dysfunction is known to induce mitochondrial mass in specific muscle fibres, RRFs, which are used as a diagnostic marker of mitochondrial disorders. These observations show that mitochondrial mass is under spatio-temporal regulation, and a complex regulatory circuit is needed to coordinate the expression of the mitochondrial proteome, which consists of approximately 1200 proteins (Calvo et al., 2016). A few activators and co-activators have been identified to induce the transcription of nuclear-encoded mitochondrial proteins, and these regulators include peroxisome proliferator-activated factors (PPARs), nuclear respiratory factors (NRFs) and estrogen-related receptors (ESRRs). ESRRs family members, ESRRA and ESRRB, resemble the estrogen receptor but do not bind estrogen or other ligands but regulate the expression of respiratory complex subunits (Mootha et al., 2004; Schreiber et al., 2004; Sladek et al., 1997).

NRF-1 and NRF-2 are TFs that are known to regulate many nuclear genes targeted to mitochondria. They were originally identified to bind to the regulatory regions of cytochrome c (NRF-1) and cytochrome c oxidase (NRF-2) subunit genes, but since then, they have been shown to have a broad role in the regulation of mitochondrial metabolism and especially in the regulation of the subunits of respiratory complexes (Evans and Scarpulla, 1989; Scarpulla, 1997, 2008). Interestingly, NRF-1 and NRF-2 also regulate the expression of human TFAM, TFB1M and TFB2M and, thereby, links the expression of nuclear and mitochondrial genomes (Gleyzer et al., 2005; Virbasius and Scarpulla, 1994). The targets of NRF-1 and NRF-2 are not, however, restricted to respiratory chain subunits or mtDNA transcription, but they also regulate mitochondrial import machinery and the assembly factor of mouse cytochrome c oxidase, COX17 (Blesa et al., 2007; Takahashi et al., 2002). Importantly, the NRFs also regulate genes with no association to mitochondrial function but encode metabolic enzymes, components of signalling pathways and gene products necessary for nucleic acid metabolism (Virbasius et al., 1993). The regulatory role of NRF-1 on these pathways is likely to underlie the embryonal lethality of NRF-1 knock-out mice (Huo and Scarpulla, 2001).

The PGC-1 family consists of three transcriptional coactivators: peroxisome proliferator-activated receptor-gamma coactivators, PGC-1α and PGC-1β, and PGC-1 related coactivator (PRC). These factors have been shown to induce mitochondrial biogenesis by binding TFs and nuclear receptors with specific DNA sequence binding properties (PGC-1 binding partners reviewed by (Villena, 2015)). The members of the PGC-1 family lack histone acetyl transferase activity, which can remodel chromatin state, but the PGC-1 factors induce gene transcription by binding and recruiting other proteins with

histone acetyl transferase activity, such as SRC-1 and CBP/p300, to the target promoters (Puigserver et al., 1999)

2.3.4.1 Expression Regulation of mtDNA Maintenance Genes

The transcription regulation of mtDNA maintenance genes is not comprehensively studied. Only few reports have characterised the genomic loci of these genes and they solely focus on *POLG* regulation.

POLG is located in chromosome 15 and is encoded by 23 exons, which span over 18.5 kilo base pair (kb) long region. The homology of the exons between mouse and human ranges from 71% - 95% (Mott et al., 2000). The promoter region is predicted to locate directly upstream from the transcription start site and this region shares 74% homology between mouse and human (Mott et al., 2000). The putative promoter does not have a TATA box sequence or predicted binding sites for known transcription regulators of mitochondrial metabolism e.g. NRF1 or PGC-1alpha (Mott et al., 2000).

POLG exon 2 has been reported to be methylated, which downregulates *POLG* expression. The methylation level was suggested to be associated with mtDNA copy number regulation (Kelly et al., 2012).

2.4 MTDNA MAINTENANCE DISORDERS

Mitochondrial dysfunction underlies a catalogue of human disorders, which can present at any age and affect many organs or show highly tissue-specific manifestation (Ylikallio and Suomalainen, 2012). The reason why specific tissues are sensitive to particular gene defects, is currently not well understood, and the question has puzzled the mitochondrial disease field for decades. The genetic background of mitochondrial disorders is diverse, and causative mutations are found in mtDNA or in nuclear genes encoding mitochondrial proteins (Wallace et al., 1988; Zeviani et al., 1990). The nuclear gene defects most commonly affect the structural proteins of respiratory chain, mitochondrial translation, proteins involved in mitochondrial dynamics or mtDNA maintenance. The focus of this thesis is mtDNA replication defects, with the emphasis on POLG and TWNK. Defects in these genes are the most common causes of mitochondrial disorders and prime examples of tissue- and genotype-specific variability of manifestations: different mutations in POLG and TWNK can either lead to severe infantile neurodegeneration or adult-onset MM. The underlying cause of these disorders is generally thought to be secondary mtDNA instability in affected tissues leading to accumulation of multiple mtDNA deletions or depletion of mtDNA copy number.

2.4.1 POLG DISORDERS

The mtDNA replicative polymerase, POLG, is the most common nuclear gene underlying mitochondrial disorders. An exceptionally large number of pathogenic mutations have been found in *POLG* and they are listed in the POLG disease mutation database (http://tools.niehs.nih.gov/polg/). POLG diseases typically manifest in the nervous system but can also affect the liver and muscle (Lamantea et al., 2002; Suomalainen and Isohanni, 2010). Despite the apparent requirement of mtDNA replication in cell division, the patients with POLG disorders do not typically show symptoms of highly proliferating cell types, such as blood or intestinal cells. The main clinical syndromes caused by POLG mutations are PEO, Alpers-Huttenlocher syndrome and mitochondrial recessive ataxia-polyneuropathy syndrome (MIRAS, MSCA-E, SANDO) (Hakonen et al., 2005; Naviaux et al., 1999; Van Goethem et al., 2001; Winterthun et al., 2005).

Alpers syndrome is typically caused by recessive POLG defects leading to tissue-specific mtDNA depletion and affects patients in early infancy (Alpers, 1931; Naviaux and Nguyen, 2004). The patients develop psychomotor retardation, liver failure and epileptic seizures typically leading to premature death before 10 years of age (Young and Copeland, 2016).

PEO can be caused by dominant or recessive POLG mutations and is characterised by accumulation of multiple mtDNA deletions in post-mitotic tissues. PEO manifests with bilateral ptosis and progressive weakening of the extraocular muscles (EOMs), generalised muscle weakness and exercise intolerance. POLG-PEO patients also present often with sensory neuropathy and early-onset parkinsonism (Luoma et al., 2004; Van Goethem et al., 2001).

POLG-related ataxia-polyneuropathy syndrome, MIRAS, typically presents in childhood or early adulthood. It is most often caused by homozygous or compound heterozygous p.W748S and p.A467T changes (Hakonen et al., 2005; Rantamaki et al., 2001; Winterthun et al., 2005). The clinical symptoms of MIRAS include gait ataxia, sensory neuropathy and some patients also develop epileptic seizures. Remarkably, patients with identical MIRAS mutations show high variability in clinical presentations indicating strong contribution of environmental or genetic modifiers.

2.4.2 TWNK DISORDERS

Dominant mutations in TWNK manifest as adult-onset PEO with progressive accumulation of multiple mtDNA deletions in patients' tissues (Spelbrink et al., 2001). Recessive TWNK defects, however, cause infantile-onset spinocerebellar ataxia (IOSCA), and the patients do not show clinical muscle symptoms (Hakonen et al., 2008; Hakonen et al., 2007; Nikali et al., 2005). IOSCA manifests after one year of age with progressive ataxia, peripheral and optic neuropathy, hearing loss and athetosis. In juvenile age, the patients develop female hypergonadotropic hypogonadism, severe epilepsy, psychiatric symptoms and hepatopathy (Koskinen et al., 1994a; Koskinen et

al., 1994b). IOSCA is the second most common inherited spinocerebellar ataxia in Finland and is caused by an ancestral homozygous founder mutation, leading to p.Y508C change (Nikali et al., 2005).

2.4.3 MTDNA DEPLETION SYNDROME

Depletion of mtDNA copy number leads to disrupted synthesis of the OXPHOS complexes causing tissue-specific or multi-tissue clinical manifestations (Moraes et al., 1991; Suomalainen and Isohanni, 2010). This genetically heterogeneous group of disorders is generally called mtDNA depletion syndrome (MDS), which is a common cause of childhood respiratory chain deficiencies (Sarzi et al., 2007). To date, 11 genes have been reported to underlie MDS, and the genetic background has provided insight into the mechanisms essential for mtDNA maintenance. No evidence is invoking reduced mtDNA half-life in MDS suggesting that the depleted mtDNA copy number is caused by insufficient mtDNA replication.

Many genes underlying MDS are directly involved in maintaining the cellular dNTP pools. These genes are thymidine kinase 2 (*TK2*), deoxyguanosine kinase (*DGUOK*), ribonucleotide reductase regulatory TP53 inducible subunit M2B (*RRM2B*) and thymidine phosphorylase (*TYMP*) (Bourdon et al., 2007; Mandel et al., 2001; Nishino et al., 1999; Papadimitriou et al., 1998; Saada et al., 2001). Defects in these genes are thought to compromise dNTP *de novo* synthesis or dNTP salvage pathways affecting total cellular and mitochondrial dNTP pools (Spinazzola et al., 2009). *TYMP* mutations are suggested to increase the cellular dTTP pool causing indirect deoxycytidine triphosphate dCTP depletion and, thereby, accounting for the mtDNA depletion (Lopez et al., 2009).

Mutations in ATP-specific succinyl-CoA synthethase beta (*SUCLA2*) and GTP-specific succinyl-CoA synthethase beta (*SUCLG2*) are also likely to cause MDS by defects in dNTP pool maintenance since they are part of the succinyl coA synthase complex, which associates with mitochondrial nucleoside diphosphate kinase (Elpeleg et al., 2005; Ostergaard et al., 2007). Recently, a novel MDS gene was discovered when mutations in *ABAT* were found from MDS patients (Besse et al., 2015). *ABAT* encodes 4-aminobutyrate aminotransferase, which physically interacts with SUCLG1, SUCLG2 and SUCLA2. The mtDNA depletion caused by *ABAT* mutations can be rescued by dNTP supplementation in cultured cells suggesting insufficient dNTP concentration in ABAT patient cell lines (Besse et al., 2015).

The most recently discovered genetic defect underlying MDS was found from patients manifesting with a severe myopathy and lactic acidosis in early childhood. These patients carry *de novo* dominant mutations in adenine nucleotide translocator (*SLC25A4*) (Thompson et al., 2016). The mutations locate in the conserved functional domain of the protein, whereas the earlier reported mutations in *SLC25A4* causing autosomal dominant PEO are found from non-conserved region of *SLC25A4* and cause a less severe enzyme defect.

Three additional genes are known to cause MDS, but they are not directly involved in dNTP pool maintenance. These genes are *POLG*, *TWNK* and *MPV17*. MPV17 is a mitochondrial protein with an unknown function, but its mutations are identified from MDS families. Interestingly, a recent study of MPV17 knock-out mice found low dTTP and dGTP pools in liver associated with mtDNA depletion and slow mtDNA replication (Dalla Rosa et al., 2016). These findings were also replicated in patient fibroblasts indicating that MPV17 is essential for dNTP pool maintenance through an unknown mechanism. POLG and TWNK are the key enzymes in mtDNA replication catalysing the synthesis of mtDNA and unwinding mtDNA double helix structure, respectively (Hakonen et al., 2008; Naviaux and Nguyen, 2004). POLG and TWNK defects are thought to be direct synthesis defects, but whether they cause secondary dNTP pool disturbance, has not been experimentally addressed.

The two mechanisms behind MDS, decreased dNTP availability and defective mtDNA synthesis, are generally accepted models for mtDNA depletion. MDS most commonly causes mtDNA depletion only in post-mitotic tissues where mtDNA is actively synthesised, but nuclear DNA is not replicating. Post-mitotic cells, therefore, synthesise dNTPs mainly to support the replication of the mitochondrial genome, which requires much less dNTPs than nuclear DNA synthesis. This sensitises post-mitotic cells to defects in dNTP synthesis enzymes which underlie mtDNA depletion.

2.4.4 THE GENETIC BACKGROUND OF MTDNA DELETIONS

Accumulation of mtDNA deletions is another underlying cause of mitochondrial disorders. Single mtDNA deletions are typically sporadic or maternally inherited, and they are always heteroplasmic. The proportion of single deletions varies between tissues and can change over time. More typically, a genetic defect leads to the accumulation of multiple mtDNA deletions. The deletions commonly occur in the region between the origins of replication of the light and heavy strands (Samuels et al., 2004). The genes underlying human disorders with mtDNA deletions are heterogeneous and partially overlapping with genes causing mtDNA depletion described above. The genetic background of mtDNA deletion syndromes are summarised in Table 1.

Table 1. *Gene defects underlying mtDNA deletions in human disorders. The table is adapted from (Viscomi and Zeviani, 2017). Abbreviations: ad, autosomal dominant; ar, autosomal recessive*

Gene Defect	Inheritance	Clinical syndrome	Reference
POLG	ad/ar	PEO/MIRAS	(Van Goethem et al., 2001), (Winterthun et al., 2005), (Hakonen et al., 2005)
POLG2	ad	PEO	(Longley et al., 2006)
TWNK	ad/ar	PEO	(Spelbrink et al., 2001)
OPA1	ad	PEO	(Amati-Bonneau et al., 2008), (Hudson et al., 2008)
RRM2B	ad	PEO	(Tyynismaa et al., 2009)
SLC25A4	ad	PEO	(Kaukonen et al., 2000)
TYMP	ar	MNGIE	(Nishino et al., 1999)
MGME1	ar	PEO	(Kornblum et al., 2013)
DNA2	ar	PEO	(Ronchi et al., 2013)
GFER	ar	myopathy	(Di Fonzo et al., 2009)
RNASEH1	ar	PEO	(Reyes et al., 2015)
TK2	ar	PEO	(Tyynismaa et al., 2012)
SPG7	ar	PEO/ataxia	(Pfeffer et al., 2014)
AFG3L2	ad	PEO/ataxia	(Gorman et al., 2015)
DGUOK	ar	PEO	(Ronchi et al., 2012)
MPV17	ar	PEO/ leukoencephalopathy	(Blakely et al., 2012)

2.5 MOUSE MODELS WITH COMPROMISED MTDNA INTEGRITY

2.5.1 DELETOR MOUSE – MODEL FOR MITOCHONDRIAL MYOPATHY

Deletor mouse is a model for mitochondrial myopathy (MM). The mice carry a TWNK transgene under the human *ACTB* promoter ubiquitously expressing a dominant TWNK patient mutation, duplication of amino acids 353-365 (Tyynismaa et al., 2005). The TWNK defect leads to generation and progressive accumulation of mtDNA deletions in muscle and brain starting at 12 months of age. This is coupled with abnormal mitochondrial ultrastructure

and cytochrome c oxidase (COX)-deficiency in 5-10% of Deletor muscle fibers by the age of 24 months (Tyynismaa et al., 2005). These key features replicate the signs of the patient disease making Deletor mouse an invaluable tool for studying the mechanisms of MM with multiple mtDNA deletions (Suomalainen et al., 1992; Zeviani et al., 1989).

The progressive respiratory chain deficiency in Deletor mice is suggested to compromise the oxidation of NADH to NAD^+ causing decreased cellular NAD^+ concentration (Khan et al., 2014). To correct this, Khan et al. supplemented the mice with an NAD^+ precursor, nicotinamide riboside (NR), which increased the cellular NAD^+ levels and reverted the disease phenotype. The treatment decreased mtDNA deletion load, decreased the number of COX-deficient muscle fibers and rescued the abnormal mitochondrial ultrastructure (Khan et al., 2014). The beneficial effect of NR is thought to be mediated by SIRT1 activation, which increases mitochondrial biogenesis. SIRT1 monitors the cellular NAD^+/NADH ratio and deacetylates FOXO1 when cellular NAD^+/NADH ratio is increased (Canto et al., 2012; Rodgers et al., 2005). This activates mitochondrial biogenesis, fatty acid oxidation and mitochondrial ATP production. Other studies have also reported beneficial effects of NR supplementation in mouse models with mitochondrial dysfunction: NR alleviated muscle pathology in a mouse model which carries p.W129K change in SCO2 corresponding to the patient p.E140K mutation (Cerutti et al., 2014; Yang et al., 2010). Together, these studies suggest that NR supplementation has high potential in treatment of MM.

The mtDNA replication defect and consequent mtDNA deletions in Deletor mice also induce a transcriptional response in the nucleus, which potentially modifies disease progression by activating or deactivating intracellular metabolic pathways and by causing systemic metabolic changes (Tyynismaa et al., 2010). One gene that shows increased expression in Deletor muscle is *Fgf21*, which is a hormone that was first identified to be secreted from liver during fasting to mobilise lipids from white adipose tissue (Badman et al., 2007; Kharitonenkov et al., 2005). In mice with MM, however, FGF21 can also be secreted from muscle (Kim et al., 2013; Tyynismaa et al., 2010). Consistent with FGF21 effects on body composition, increased circulating FGF21 in Deletors is associated with decreased body fat and reduced liver fat content (Tyynismaa et al., 2010; Tyynismaa et al., 2011).

FGF21 is also increased in the plasma of PEO patients and other mitochondrial patient groups, and it was further shown to be a sensitive and specific marker for mitochondrial disease (Davis et al., 2013; Fujita et al., 2015; Salehi et al., 2013; Suomalainen et al., 2011). Recently, increased plasma FGF21 level was associated to gene defects causing mitochondrial translation problem but not to defects that cause primary respiratory chain dysfunction (Lehtonen et al., 2016).

2.5.2 MUTATOR MOUSE

POLG is a high-fidelity polymerase due to its exonuclease activity, which recognises falsely inserted nucleotide during replication and excises it before replication can continue. This ensures faithful replication of mtDNA and prevents transmission of mutations to the daughter molecule. Abolishing the POLG exonuclease function in mice induces generation and accumulation of mtDNA point mutations in all tissues leading to a premature ageing-like phenotype (Trifunovic et al., 2004). These knock-in "Mutator" mice manifest with kyphosis, osteoporosis, low body weight, decreased adipose tissue and thinning of the skin. The mice also develop progressive megaloblastic anaemia leading to early death around 13-15 months.

The Mutator mouse, together with an extensive field of research, has discounted the mitochondrial theory of ageing which states that the generation of mtDNA point mutations potentially leads to a vicious cycle by inducing ROS generation (Sanz, 2016). ROS is a potent DNA damaging agent which could induce further generation of mtDNA lesions. The Mutator mouse, however, does not show exponential generation of point mutations, but the driving force behind the ageing phenotype of the mouse is thought to be dysfunctional respiratory chain or defective mitochondrial translation (Edgar et al., 2009).

2.5.3 MNGIE MOUSE

Defects in genes critical for dNTP pool maintenance underlie a spectrum of mitochondrial disorders. One of these genes is *TYMP* whose mutations cause mtDNA deletions and depletion manifesting as mitochondrial neurogastrointestinal encephalopathy (MNGIE) (Nishino et al., 1999). TYMP degrades cellular dNTPs, and the loss of its activity causes increased thymidine (Thd) and deoxyuridine (dUrd) levels leading to dNTP pool imbalance and consequent loss of mtDNA integrity. TYMP knockout mouse ($TYMP^{-/-}$) was created to study the MNGIE disease mechanisms and to test putative treatment strategies (Lopez et al., 2009). Since murine uridine phosphorylase (UPP1) can compensate for the loss of TYMP activity and degrade Thd and dUrd, ($TYMP^{-/-}$) mouse was crossed with UPP1 knock-out mice ($UPP1^{-/-}$) to obtain TYMP/UPP1 double knockout ($TYMP^{-/-}$, $UPP1^{-/-}$) mice. The ($TYMP^{-/-}$, $UPP1^{-/-}$) mice recapitulate features of MNGIE patients and have increased Thd and dUrd levels in several tissues, including brain, muscle and small intestine (Lopez et al., 2009). The increased Thd and dUrd levels lead to increased dTTP and decreased dCTP levels in the brain, which is thought to underlie the progressive loss of mtDNA copy number. The mice have 27% mtDNA depletion in brain at the age of 6 months and 61% depletion at 14-18 months, but no mtDNA deletions were detected.

The elevation of Thd and dUrd in tissues of ($TYMP^{-/-}$, $UPP1^{-/-}$) mice is relatively modest (<65-fold) compared to MNGIE patients (>100-fold). This might explain the milder phenotype of the mice affecting mostly brain. Stressing the mice with long-term oral Thd and dUrd supplementation further

elevated their levels exacerbating the disease phenotype (Garcia-Diaz et al., 2014). The treatment reduced the survival, body weight and motor functions of the mice and induced mtDNA depletion in the small intestine, which was accompanied with the loss of smooth muscle cells of the muscularis proparia (Garcia-Diaz et al., 2014).

The dNTP pool imbalance in the ($TYMP^{-/-}$, $UPP1^{-/-}$) mice also leads to generation of mtDNA point mutations as the authors reported increased levels of C-to-T transitions in mtDNA, which have previously been observed also in MNGIE patients (Nishigaki et al., 2003). The C-to-T transitions were detected frequently after a stretch of A residues, which indicates that they are induced by next-nucleotide effect. This suggests that dNTP imbalance can lead to generation of mtDNA point mutations even with intact POLG exonuclease activity (Lopez et al., 2009; Nishigaki et al., 2003).

3 AIMS OF THE STUDY

The general aim of this thesis was to understand the molecular pathology and tissue-specificity of mtDNA maintenance diseases. The specific aims were as follows:

To identify and characterise the metabolic response pathways in mitochondrial disease patients and mouse models with mtDNA replication defects.

To find new treatment strategies for mtDNA maintenance disorders by modifying metabolic response pathways.

To study the transcription regulation of mtDNA maintenance genes by searching for genomic non-coding regulatory elements.

4 MATERIALS AND METHODS

All methods are described in detail in the original publications and briefly presented here.

4.1 ETHICAL STATEMENTS AND ANIMAL LICENSES

The Ethical Review Board of Helsinki University Central Hospital approved the use and collection of human samples. The National Animal Review Board and Regional State Administrative Agency for Southern Finland approved the animal experiments used in this thesis, and they were conducted according to the European Union Directive. The animal experiments were performed under the following license number: ESAVI/689/04.10.07/2015.

4.2 ANIMAL MODELS

Deletor mice were previously described in (Tyynismaa et al., 2005). The mice express a dominant mutant TWNK with an in-frame duplication of amino acids 353-365 under ubiquitous *ACTB* promoter. The transgenic mice were maintained in C57BL/6 background.

IOSCA mice were created by introducing the point mutation c.1526 A>G (p.Y509C) into a vector construct having frt-flanked neomycin (NEO) selection cassette together with homologous arms flanking the targeted region. The construct was electroporated into Sv129 mouse embryonic stem (ES) cells and introduced to the genome by homologous recombination. The positive ES cells were injected into C57BL/6 blastocysts. The NEO cassette was removed by crossing the IOSCA mouse with a mouse ubiquitously expressing Flp recombinase. The resulting mice were backcrossed to C57BL/6 by using speed congenic method utilising whole-genome single-nucleotide polymorphism (SNP) analysis.

Enhancer regions and 250-300 bp flanking regions were amplified by polymerase chain reaction (PCR) and subsequently cloned into tkPD destination vector (gift from Jussi Taipale). The resulting constructs were linearised and injected into mouse zygotes to generate the enhancer founder lines. *Polg* promoter construct was created by cloning a region of 500 bp upstream from *Polg* transcription start site into tkPD vector without the minimal HSV-tk promoter. The primer sequences used for cloning the constructs can be found from the original articles. Staining patterns were considered real if the same pattern was detected in 3 founder lines.

4.3 HISTOLOGICAL STAININGS

Enzyme activity stainings (COX and SDH) were performed as described in (Ahola-Erkkila et al., 2010). Freshly collected muscle samples were embedded with Tissue-Tek O.C.T.™ compound and frozen in isopentane cooled with liquid nitrogen.

For immunohistochemistry and immunofluorescent staining, tissue-samples were embedded into paraffin and sectioned. Paraffin was remove, and the tissue sections stained with standard protocols by using the following primary antibodies: pS6 (Cell signalling, #2217), Calbindin (Abcam, AB11426), β-Gal (Abcam, AB9361) and Islet1/2 (gift from Juha Partanen).

4.4 MITOCHONDRIAL DNA ANALYSIS

Total DNA was extracted by using phenol/chloroform extraction. The mtDNA copy number was analysed by quantitative PCR using SYBR Green and primers as in (Khan et al., 2014). MtDNA deletion load was analysed by triplex qPCR assay with 5' modified probes with ROX (D-Loop probe), HEX (ND1 probe) or FAM (ND4 probe) for the use in single-well detection (Rygiel et al., 2015).

4.5 ENHANCER ELEMENT PREDICTIONS

Enhancer Element Locator version 1.5.2.2 was used in the predictions of enhancer elements. The coding region and 100 kb upstream and downstream of mtDNA maintenance genes was analysed in human, rat and mouse. Transcription factor binding site matrices provided by the program with added matrices for AHRARNT.01 and NFX_ARNT.01 (Genomatix) were the base of the analysis.

4.6 *LACZ* STAINING

Adult mice were perfused with ice-cold phosphate buffered saline (PBS) before tissue dissection. Mouse embryos were collected at E12.5, immersed in ice-cold PBS and immediately dissected and washed once with phosphate buffer (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4). The embryos were fixed in fixing solution (0.2% glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$ in 0.1 M phosphate buffer) for 30 minutes at room temperature. Fixed samples were washed 3 x 15 minutes in washing buffer (2 mM $MgCl_2$, 0.01% DOC, 0.02% Nonidet P-40, 0.1 M phosphate buffer) and stained in X-Gal staining solution (5 mM ferrocyanide, 5 mM ferricyanide, 1 mg/ml X-gal in washing buffer) at 37°C upon agitation until desired intensity was reached (minimum 3h). The stained

samples were rinsed 3 times with washing buffer and fixed in 4% paraformaldehyde (PFA) overnight at 4°C.

4.7 METABOLOMICS ANALYSIS

Metabolite measurements ("100-metabolites" and folate intermediates) were performed by Waters Acquity ultra performance liquid chromatography (UPLC) with triple-quadruple mass spectrometry (MS) detection. Detailed instrumentation and analytical conditions are described in the original articles.

4.8 IN VIVO [U-^{13}C]-GLUCOSE FLUX

20 mg of [U-^{13}C]-glucose (Cambridge Isotope Laboratories, catalogue no. CLM-1396) was administered to Deletor and WT mice via tail vein injection. The mice were sacrificed and tissues collected at 15 minutes. The samples were analysed by targeted selected reaction monitoring (SRM) liquid chromatography-tandem mass spectrometry [LC-MS/MS] on an Agilent 6460 QQQ instrument.

4.9 [^{18}F]-FDG AND [^{18}F]-FOLATE TRACER EXPERIMENTS

Food was removed from mice four hours before the *in vivo* tracer experiments. The mice were anesthetised with isoflurane and a catheter was placed to the tail vein 15 minutes before intravenous administration of 5 MBq of [^{18}F]-FDG or [^{18}F]-folate. The mice were kept anesthetised for 60 minutes on temperature-controlled mats, and the activity of tissues was measured with a gamma counter.

4.10 DNTP POOL MEASUREMENTS

DNTP pools were measured from whole-tissue lysates using a sensitive polymerase-based assay. Detailed description of the method is described in (Nikkanen et al., 2016).

4.11 QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from samples using Trizol reagent (Life Technologies, #15596-026). 1 µg of RNA was used in complementary DNA (cDNA) synthesis (Maxima First Strand cDNA synthesis kit, Thermo

Scientific, #K1672). Quantitative amplification of the target cDNAs was performed using iQ Sybr Green (Bio-Rad, #172-5006) on a CFX96 Touch Real-Time PCR System (Bio-Rad). *Actb* was used as a reference gene to control for equal cDNA amount used in reactions.

4.12 WESTERN BLOT

Tissues were homogenised with Precellys bead homogeniser, and total protein extracted with 1% Triton-X in 50 mM Tris, 150 mM NaCl buffer, pH 7.6. Proteins were separated with SDS-PAGE, blotted on PVDF membrane and visualised using the following primary antibodies incubated overnight at 4°C: CTH (Proteintech,12217-1-AP), SDHA (Abcam, ab14715), ACTB (Santa Cruz, sc1616), alpha Tubulin (Santa Cruz Cat #sc-5286) and MTHFD2 (Abcam, #37840).

4.13 DNASE I HYPERSENSITIVE SITE ANALYSIS

Distal regulatory DHSs were identified using public data deposition from DNase I -seq experiments (Thurman et al., 2012). A DHS was considered a likely regulatory site of the target gene if it located <500 kb from the coding region, and its sensitivity to DNase I treatment correlated >0.85 with the sensitivity of a DHS in the target promoter across analysed samples.

4.14 STATISTICAL ANALYSES

All statistical testing was performed by using Student's t-test or ANOVA where indicated.

Outliers were removed from the metabolomics data using ROUT method (Q = 1%) (GraphPad Prism 6.0). False discovery rate was controlled using Benjamini-Hochberg method with the critical value of 0.2.

PCA plots were created in Metaboanalyst 2.0 with autoscaled data. Missing values were estimated using KNN method.

4.15 CELL CULTURE EXPERIMENTS

Human myoblasts were treated with actinonin for 16 hours before analysis. *ATF4* silencing was performed with SilencerSelect© oligos (Sigma) according to the instructions of the manufacturer.

5 RESULTS AND DISCUSSION

5.1 MITOCHONDRIAL REPLICATION DEFECTS MODIFY CELLULAR DNTP POOLS, ONE-CARBON METABOLISM AND GLUCOSE CONSUMPTION (I)

5.1.1 MITOCHONDRIAL MYOPATHY INCREASES GLUCOSE UPTAKE FOR *DE NOVO* SERINE SYNTHESIS

In study I, we aimed to characterise the metabolic consequences of mtDNA replication defects by using a mouse model for mitochondrial myopathy (MM), the Deletor mouse. These mice carry a dominant TWNK defect, and they faithfully replicate the human disease by accumulating mtDNA deletions and COX-deficient muscle fibers (Tyynismaa et al., 2005). We also found mtDNA deletions in the heart and consequent COX-deficient and SDH-positive cardiomyocytes (Figure 7).

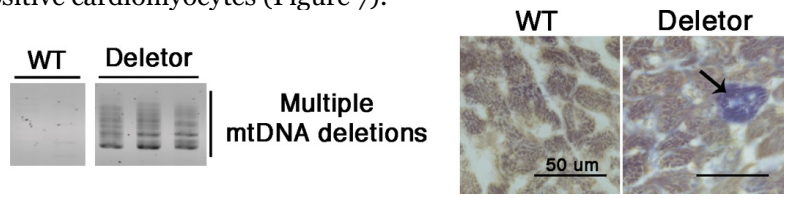

Figure 7 Left, multiple mtDNA deletions in Deletor heart (PCR amplification). Right, enzyme activity staining (COX, brown; SDH, blue) from the heart of WT and Deletor mice. COX-deficient and SDH-positive cardiomyocyte found from Deletor heart (black arrow). Scale bars 50 µm.

Metabolic profiling of the muscle and heart of the Deletor mice was performed by measuring the concentration of 107 metabolites by UPLC/MS. This revealed a genotype-specific metabolic fingerprint due to mitochondrial dysfunction (Figure 8). Individual quantifications showed that increased steady-state serine level was the most significant change in both tissues (Figure 8). Interestingly, we have previously found that Deletor muscle upregulates the expression of phosphoglycerate dehydrogenase (PHGDH) and phosphoserine aminotransferase 1 (PSAT1), which are enzymes that catalyse the first two steps in *de novo* serine synthesis (Figure 8) (Tyynismaa et al., 2010). These enzymes are part of the transcriptional response which increases the expression of genes with an amino acid response element in their promoters (Tyynismaa et al., 2010). The induced expression of serine biosynthesis enzymes and increased serine steady-state level suggested activated serine biosynthesis in Deletor muscle and heart.

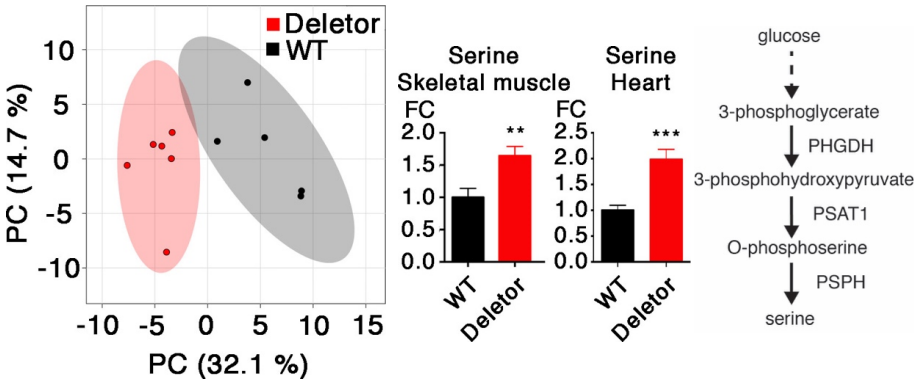

Figure 8 Left, principal component analysis of metabolomics data from skeletal muscle of WT and Deletor mice. Plot shows genotype-specific separation of the groups. Middle, quantification of serine levels in skeletal muscle and heart. Right, serine biosynthesis pathway.

Serine is synthesised *de novo* from glucose, and we found a highly increased *in vivo* 2-[^{18}F]-fluoro-2-deoxy-D-glucose ([^{18}F]-FDG) uptake in the muscle and heart of the Deletor mice (Figure 9). To test if glucose is directed into serine biosynthesis, we administered [U-^{13}C]-glucose to Deletor and WT mice via tail vein injections and followed the incorporation of glucose-derived carbons into serine after a 15-minute chase (Figure 10). This time point is in the linear range of serine synthesis (Figure 10). The fully [^{13}C]-labelled serine (m+3) was significantly increased in Deletor heart compared to WT mice showing induction of *de novo* serine synthesis (Figure 10).

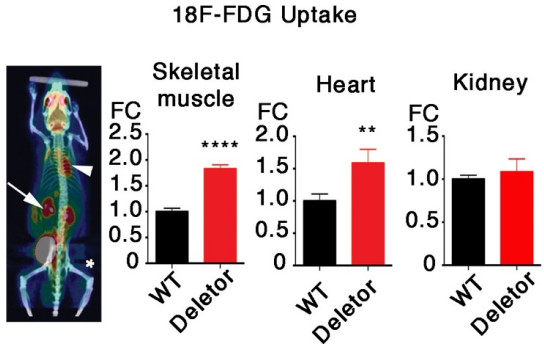

Figure 9 *In vivo* [^{18}F]-FDG uptake in skeletal muscle (star), heart (white arrowhead) and kidney (white arrow). Right, quantifications of the uptake in tissues.

PHGDH is a metabolic gate-keeper which can divert glucose consumption from glycolysis into serine synthesis. For example, cancer cells induce the expression of PHGDH to synthesise serine for cellular growth (Possemato et al., 2011). Flux experiments in cultured cells with labelled glucose have indicated that glucose carbons are incorporated into serine and lactate in equal rates implying that serine is a major glucose-consuming pathway (Locasale et al., 2011). Therefore, our results suggest that the increased glucose uptake in mitochondrial diseases supports serine synthesis and might not be a compensatory response to generate ATP through glycolysis.

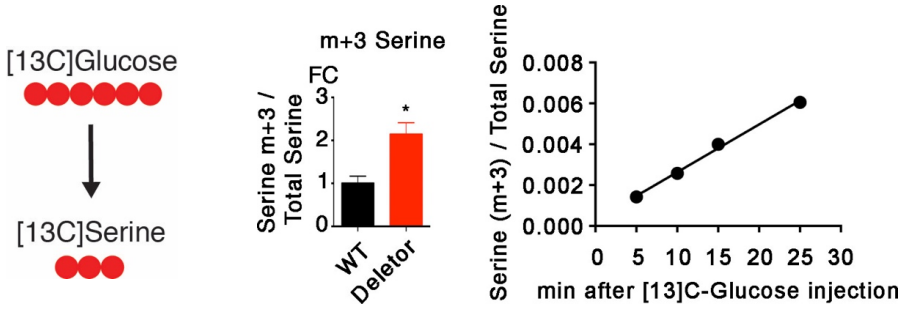

Figure 10 Left, 13C-labelled glucose carbons are incorporated into the three-carbon backbone of serine. Middle, quantification of m+3 serine in the heart of WT and Deletor mice after administration of [U-^{13}C]-glucose, chase for 15 minutes. Right, labelling of m+3 serine in heart in the function of time.

5.1.2 SERINE SYNTHESIS SUPPORTS INCREASED TRANSSULFURATION

Since serine can serve as a substrate in multiple cellular reactions, we next sought an explanation for the increased serine synthesis in Deletor mice. We searched for genes that share evolutionary history with PHGDH and are, therefore, likely to be linked to *de novo* serine synthesis. Functionally related gene groups are typically preserved together in evolution, and this has recently been shown to be a powerful trait when predicting functionally interdependent pathways (Li et al., 2014). The top five hits for PHGDH include cystathionine beta synthase (CBS) and cystathionine gamma lyase (CTH), which are the first two enzymes in the transsulfuration pathway (Figures 11, 12). Interestingly, CBS uses serine and homocysteine as substrates to produce cystathionine, the level of which was increased in the heart of Deletor mice. We next measured the expression levels of CBS and CTH and found highly induced expression of CTH in muscle and heart, but the level of CBS was not detectable by western blot in WT or Deletor mice (Figure 11).

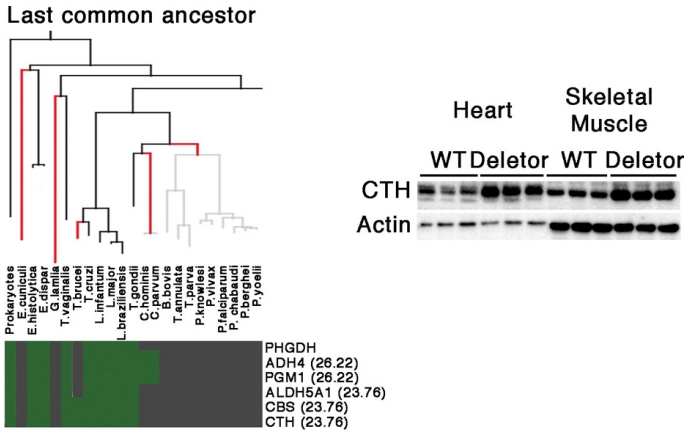

Figure 11 Left, genes co-evolving together with PHGDH (CLIME analysis). Scores in brackets indicate CLIME-scores. Red lines show species that have lost PHGDH. Figure modified from CLIME. Right, expression of CTH in the heart and skeletal muscle of WT and Deletor mice (western blot).

Transsulfuration pathway produces cysteine, which is an amino acid used in protein synthesis and metabolic pathways. Cysteine is the rate-limiting metabolite in glutathione synthesis, which is a major cysteine-dependent pathway (Lu, 2013). Elevated expression of GCLC, a catalytic subunit of the first step in glutathione synthesis, together with the higher concentration of gamma-glutamylcysteine in the Deletor heart indicated increased glutathione synthesis (Figure 12).

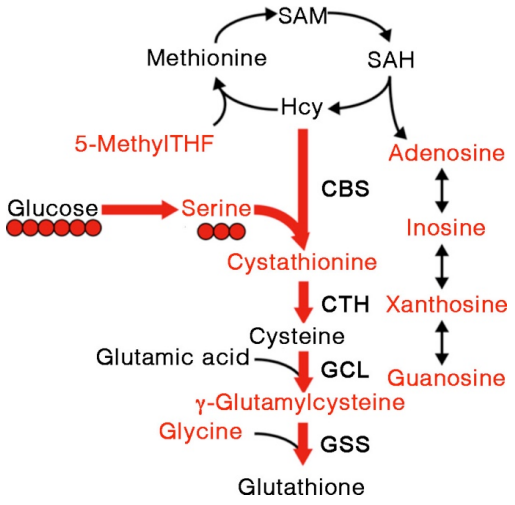

Figure 12 Schematic picture of methyl cycle, serine biosynthesis, transsulfuration and glutathione synthesis. Picture summarises the findings from Deletor heart: increased metabolites shown in red.

Glutathione is a key metabolite in nucleotide metabolism as it forms the core in the hydrogen donor system, which provides hydrogen molecules for ribonucleotide reductases in dNTP synthesis (Sengupta and Holmgren, 2014). The role of glutathione in mitochondrial dysfunction has been widely studied, but the essential role of glutathione in dNTP synthesis has commonly been overlooked because of its important role in ROS scavenging, which is often associated with mitochondrial dysfunction (Atkuri et al., 2009). Intriguingly, induced *de novo* serine synthesis for glutathione production has recently been shown to be essential in cancer cells due to the high need of dNTPs in cell proliferation (DeNicola et al., 2015). In quiescent cells dNTPs are used for mtDNA maintenance, and therefore, we hypothesised that mtDNA replication stress might induce dNTP synthesis in post-mitotic tissues and result in the detected increased serine-driven glutathione production (Bourdon et al., 2007). We measured the levels of dNTPs from Deletor muscle lysates and detected increased levels of all four pools dATP, dTTP, dGTP and dCTP. Increased and imbalanced dNTP pools are directly mutagenic and compromise the integrity of both mitochondrial and nuclear genomes (Garcia-Diaz et al., 2014). Therefore, the metabolic response increasing dNTP pools is a likely contributor to the mtDNA mutagenesis in the Deletor mouse.

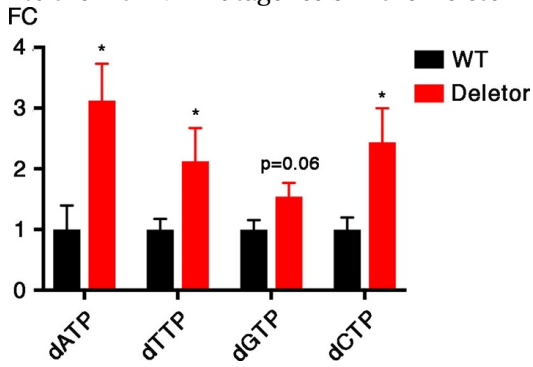

Figure 13 The levels of dNTP pools in skeletal muscle of WT and Deletor mice. Abbreviations: FC, fold change.

5.1.3 MITOCHONDRIAL DISEASE REMODELS ONE-CARBON METABOLISM

Nucleosides are the precursors for dNTPs, and they are produced *de novo* from amino acids and ribose-5-phosphate via purine and pyrimidine synthesis pathways. *De novo* purine synthesis and the conversion of dUMP to dTMP require carbon units to be incorporated to their nitrogenous bases. These carbons are provided by the folate cycle, which is a metabolic pathway that uses amino acids as input and donates 1C-units to nucleoside synthesis and to other key metabolic processes reviewed above (Tibbetts and Appling, 2010). Interestingly, Deletor muscle and heart showed increased protein expression of MTHFD2 and MTHFD1L, which are the enzymes of the mitochondrial folate

cycle, which produces formate for *de novo* purine synthesis (Figure 14). *Mthfd2* was earlier found to be a part of the transcriptional response in Deletors together with serine biosynthesis enzymes (Tyynismaa et al., 2010). These changes, together with induced dNTP levels, predicted changes in the folate cycle, which prompted us to characterise the folate metabolism in the Deletor mouse.

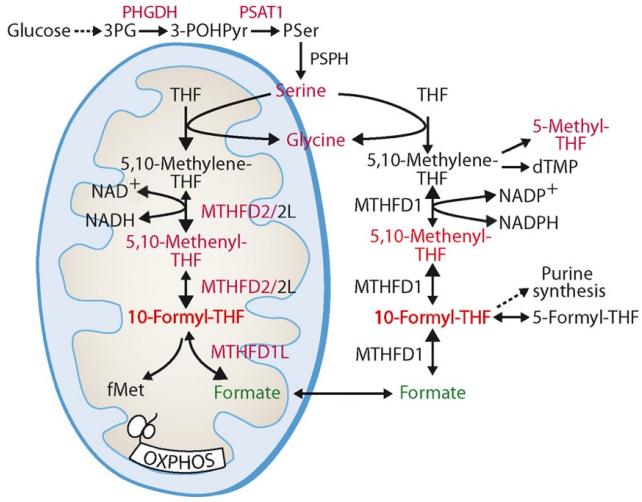

Figure 14 Schematic picture of serine synthesis and 1C-metabolism. Picture summarises the findings from Deletor skeletal muscle. Red indicates increased level of expression/concentration. Formate in green indicates reduced concentration. Metabolites or enzymes with black font were not changed or not measured.

The activation of the mitochondrial folate cycle was coupled with active [^{18}F]-folate uptake by the Deletor muscle increasing THF, 5-methyl-THF and methylene-THF levels (Figure 14). When the mice were supplemented with folinic acid (5-formyl-THF), Deletor muscle showed a drastic increase in 5/10-formyl-THF pool, whereas only a modest increase in the WT animals was detected (Deletors 6-fold increase, WT 2-fold increase). These data suggest that mitochondrial myopathy modifies the demand of folate in the affected tissue. However, folinic acid supplementation did not ameliorate disease pathogenesis (the number of COX-deficient muscle fibres, mtDNA deletion load) nor affect the expression of the transcription response genes *Mthfd2*, *Fgf21*, *Psat1* or *Phgdh*. These data indicated that the pathological changes of MM are not improved by increased nutritional folate availability.

An important 1C-carrier for purine synthesis is the cytoplasmic 10-formyl-THF, which is dependent on formate production by mitochondria. Formate is produced in the mitochondria from methylene-THF by a series of reactions catalysed by MTHFD2/2L and MTHFD1L (Figure 14). Deletor muscle contained reduced formate concentration ($p<0.0001$) indicating either an induced utilisation or defective synthesis. The synthesis requires the conversion of methylene-THF to 10-formyl-THF by MTHFD2, which is

dependent on NAD^+ cofactor. A study in HEK293 cells with mtDNA depletion, induced by a dominant negative POLG mutation, reported increased expression of MTHFD2 and activation of analogous stress response to Deletor muscle (Bao et al., 2016). The authors concluded that in their model MTHFD2 catalyses a reverse reaction producing methylene-THF leading to impaired mitochondrial formate production due to low NAD^+ level (Bao et al., 2016). In Deletors, the reoxidation of NADH is impaired leading to low NAD^+ level, which might also underlie defective formate production (Khan et al., 2014). However, reports from the cancer field have shown that mitochondrial folate pathway is activated due to the induction of the formate-dependent purine synthesis (Ben-Sahra et al., 2016). These observations indicate a context-dependent function of MTHFD2 and mitochondrial folate cycle. The role of MTHFD2 in the muscle of Deletor mice remain unclear until further *in vivo* flux and knock-down experiments.

An alternative reason for the induced mitochondrial folate cycle in Deletor muscle might be nicotinamide adenine phosphate (NADPH) production since NADPH can be produced from mitochondrial and cytoplasmic 10-formyl-THF (Fan et al., 2014). Increased glutathione synthesis, together with possible NADPH production, in Deletors point toward a hypothesis that folate cycle can act as a NADPH-producing stress pathway to ensure efficient reduction of oxidised glutathione.

5.1.4 MOUSE MODEL FOR IOSCA REPLICATES THE HUMAN DISEASE

We created another mouse model with a TWNK defect to see if the identified metabolic stress pathways have relevance for other mtDNA maintenance disorders. This mouse carries a recessive IOSCA mutation (c. 1526 A>G, p.Y509C), which causes severe neurodegeneration in humans. The mutation was introduced into exon 3 of *Twnk* by homologous recombination. Correct recombination of the targeting construct into the mouse genome was verified by southern blotting.

The mice were born in Mendelian proportions and weighed significantly less starting from 9 months of age. A subset of male mice (5/21) had epileptic seizures induced by handling. Histopathological examination revealed arborisation of the apical dendritic trees of the hippocampus. However, no COX deficient muscle fibres were detected, and the quality of mtDNA was intact in the muscle and brain, but a modest reduction of mtDNA copy number was detected in liver (fold change -1.3, $p < 0.05$).

5.1.5 IOSCA MUTATION DOES NOT INCREASE SERINE SYNTHESIS BUT LEADS TO THE LOSS OF DNTP POOLS IN MUSCLE

As Deletor mice showed metabolic reprogramming in the muscle, we examined these pathways in IOSCA mice. General reduction in amino acid

levels was detected, but no signs of induced serine synthesis, transsulfuration or remodelling of 1C-metabolism were found from the muscle. However, dNTP pools were drastically reduced in IOSCA muscle, which was an opposite finding compared to the Deletor mouse where dNTPs were increased (Figure 15).

The reduced dNTP pools were not sufficient to induce mtDNA depletion in muscle of IOSCA mice, which is consistent with other mtDNA depletion models with dNTP synthesis defects (Tyynismaa and Suomalainen, 2009). Our findings show that the IOSCA mutation modifies cellular dNTP pools, which suggests that mtDNA depletion in human IOSCA patients might be due to limited dNTP availability. This fits together with the fact that most gene defects causing mtDNA depletion compromise cellular dNTP synthesis (Suomalainen and Isohanni, 2010).

The IOSCA mouse is a valuable new model when studying the disease mechanisms in the future. Also, nucleoside treatment has been lately proposed as a treatment for dNTP pool disorders, and the IOSCA mouse is a great tool to test this intervention as a treatment for IOSCA or for mitochondrial encephalohepatopathies.

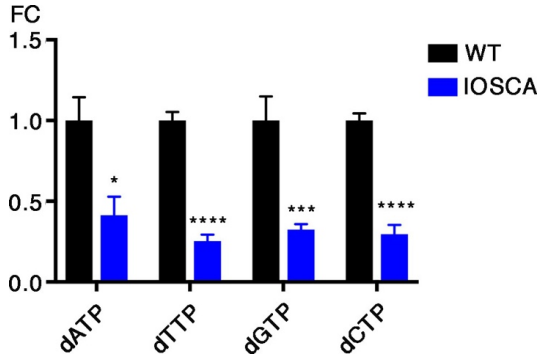

Figure 15 The level of dNTP pools in skeletal muscle of WT and IOSCA mice. Abbreviations: FC, fold change.

5.1.6 BLOOD CYSTATHIONINE IS INCREASED IN MITOCHONDRIAL DISEASE PATIENTS

To clarify if the metabolic changes in our mouse models have relevance in human patients, we performed metabolomics analysis from patient blood and muscle samples. MM patient muscle had increased concentration of serine, cystathionine and glutamate indicating that the similar response pathway as in Deletor mice is also activated in MM patients. We also found that cystathionine was increased in the blood of various mitochondrial disease syndromes (PEO, IOSCA, MELAS and MIRAS) when compared to the control subjects (PEO and IOSCA reported in (Nikkanen et al., 2016), MELAS and MIRAS is unpublished data). This indicates that metabolite biomarkers could potentially be used in the diagnosis of mitochondrial diseases together with

other novel biomarkers, such as FGF21 and GDF15 (Lehtonen et al., 2016; Suomalainen et al., 2011).

5.2 MTORC1 INDUCES METABOLIC REMODELING IN MITOCHONDRIAL MYOPATHY (II)

In study I, we found increased levels of amino acids and induction of transsulfuration and 1C-metabolism in the Deletor mouse indicating that mtDNA replication dysfunction leads to an activation of cytoplasmic anabolic pathways. This guided us to study nutrient sensing pathways in detail, and we sought an explanation for the increased anabolism in the Deletor mouse.

5.2.1 MTORC1 ACTIVITY IS INDUCED IN MITOCHONDRIAL MYOPATHY

Mechanistic (mammalian) target of rapamycin complex I (mTORC1) is responsive to cellular nutritional state and is a major regulator of cellular metabolism (Saxton and Sabatini, 2017). In fed state, mTORC1 is phosphorylated, which activates cellular anabolism and blocks catabolic pathways, e.g. autophagy. This promotes growth by inducing protein, nucleotide and lipid synthesis (Laplante and Sabatini, 2012). The overlap of the mTORC1-regulated pathways and our findings in Deletor muscle, prompted us to study mTORC1 metabolism in MM.

Phosphorylation of ribosomal protein S6 is an indication for mTORC1 activity since it is phosphorylated by S6 kinase that is a direct mTORC1 target (Ma and Blenis, 2009). When activated, mTORC1 induces cellular growth and activates protein synthesis by directly regulating the activity of cytoplasmic ribosomes (Ma and Blenis, 2009). The phosphorylation status of S6 in Deletor muscle, which was analysed by immunohistochemistry, showed a mosaic induction in single muscle fibers (Figure 16). Interestingly, the muscle fibers with the most severe respiratory chain deficiency (COX-, SDH+) showed intense pS6 staining. These data indicated that primary mitochondrial

dysfunction activates mTORC1 in skeletal muscle and prompted us to study its role in the metabolic stress response and disease pathogenesis.

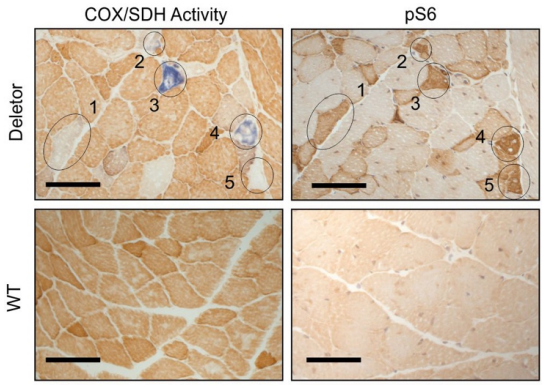

Figure 16 Enzyme activity staining (COX, brown; SDH, blue) and immunohistochemistry with anti-pS6 antibody from consecutive skeletal muscle sections. pS6-positive muscle fibers in Deletor are often COX- or COX-/SDH+ (circled fibers).

5.2.2 INHIBITION OF MTORC1 DECREASES SERINE BIOSYNTHESIS, TRANSSFULFURATION AND ONE-CARBON METABOLISM

We inhibited mTORC1 activity in 22-month-old Deletor mice by rapamycin treatment for 70 days (dose 8 mg/kg/d, intraperitoneal injections) to evaluate its role in MM. The treatment reduced the phosphorylation of S6 protein showing efficient inhibition of mTORC1.

The inhibition of mTORC1 completely abolished MTHFD2 protein expression and also reduced the *Mthfd2* messenger RNA (mRNA) level (Deletor rapa vs Deletor veh: $p<0.01$) (Figure 17). This was coupled with the reduction of folate intermediate levels to WT-levels after rapamycin treatment, which indicates that mTORC1 activation is underlying the remodelling of 1C-metabolism in Deletor mice (Figure 17). Rapamycin-treatment also rescued the induction of transsulfuration enzyme CTH and reduced the mRNA expression of *de novo* serine biosynthesis enzymes *Phgdh*, *Psat1*, and *Psph* (Figure 17) (Deletor rapa vs Deletor veh for all serine biosynthesis enzymes: $p<0.01$). The downregulation of all stress response pathways indicated that mTORC1 acts upstream of the responses. This provided an opportunity to find the regulators of the transcription circuit and to test the role of the response pathways on disease pathogenesis.

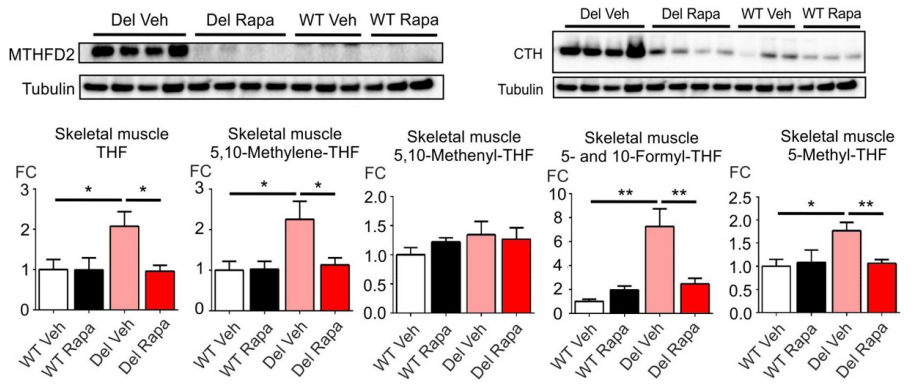

Figure 17 Upper panel, protein expression of MTHFD2 and CTH in mouse groups treated with rapamycin or vehicle (western blot). Lower panel, quantifications of folate intermediates in skeletal muscle of mouse groups in the treatment trial.

5.2.3 ATF4 INDUCES THE METABOLIC STRESS RESPONSE IN MITOCHONDRIAL MYOPATHY

The regulators that activate the transcription response in MM are unknown. In cancer, mTORC1 activation is known to regulate gene expression by stabilising ATF4 transcription factor, which binds to the target promoters (e.g. *MTHFD2)* and induces their transcription (Ben-Sahra et al., 2016). We studied the role of ATF4 in a myoblast culture system and induced mitochondrial dysfunction by actinonin treatment, which leads to upregulation of *MTHFD2* expression (Richter et al., 2015) (Figure 18). We found robust induction of *ATF4* expression after the treatment. Knocking down the expression of *ATF4* completely blocked the induction of *MTHFD2* expression, which strongly indicates that ATF4 is an upstream regulator of the transcriptional response in cultured cells. This supports the conclusion that ATF4 mediates mTORC1-regulation in MM muscle (Figure 18).

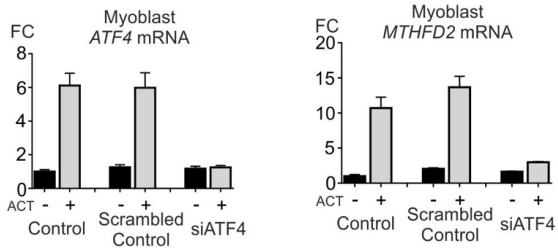

Figure 18 The mRNA expression of *ATF4* and *MTHFD2* in myoblasts after actinonin-treatment (RT-qPCR). Silencing of *ATF4* expression was done with siRNA (siATF4).

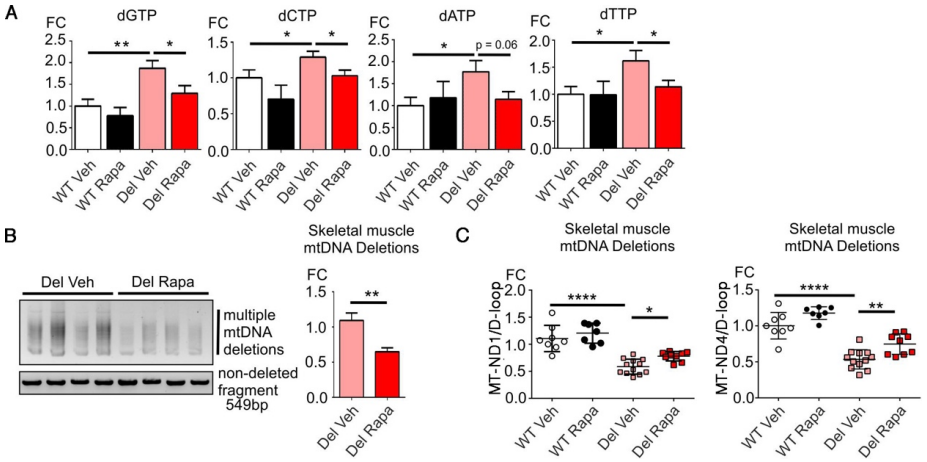

Figure 19 (A) The levels of dNTP pools in skeletal muscle of mouse groups treated with rapamycin or vehicle. (B) MtDNA deletions in Deletor skeletal muscle after rapamycin or vehicle treatment and quantification of signal, semi quantitative PCR amplification. Non-deleted fragment was amplified to control for equal DNA amount. (C) Quantitative analysis of deletion load in MT-ND1 and MT-ND4 of mouse groups treated with vehicle or rapamycin, triplex QPCR.

5.2.4 RAPAMYCIN AMELIORATES RESPIRATORY CHAIN DYSFUNCTION

As mTORC1 also regulates nucleotide metabolism, we hypothesised that mTORC1 contributes to the dNTP pool maintenance and mtDNA mutagenesis in Deletors. The increased dNTP pools were completely rescued after rapamycin-treatment, which was coupled with significant reduction in mtDNA deletions (Figure 19). The reduction of mtDNA deletion load further predicted a beneficial effect of mTORC1 inhibition on disease manifestation. Indeed, the number of both COX- and COX-/SDH+ muscle fibres were reduced (Figure 20). This was consistent with reduced circulating serum FGF21, which is a mitochondrial myopathy disease marker (Figure 20). However, no mitochondrial biogenesis or changes in the expression of respiratory chain complexes were detected, which indicates that the treatment mechanism was independent from mitochondrial biogenesis. All other previous treatment strategies for MM, e.g. NAD^+ precursors, PARP-inhibitors and ketogenic diet, have induced mitochondrial biogenesis (Ahola-Erkkila et al., 2010; Khan et al., 2014; Pirinen et al., 2014). Our data suggest that chronic induction of cellular anabolism by mTORC1 might be a key contributor in MM progression. Therefore, rapamycin treatment should be considered when designing treatment trials for MM patients.

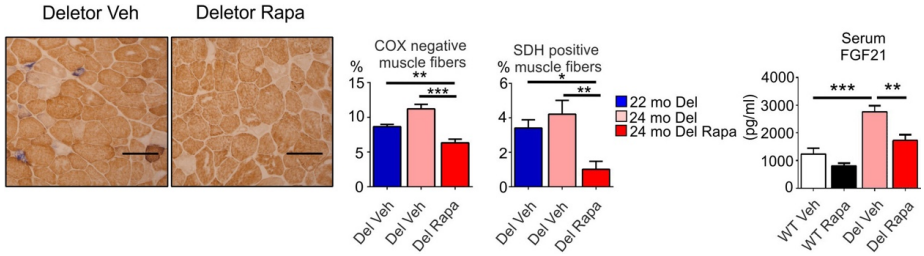

Figure 20 Left, enzyme activity staining (COX, brown; SDH, blue) of Deletor mice treated with rapamycin or vehicle. Middle, the number of COX- and SDH+ muscle fibres of Deletors. Right, circulating FGF21 level (ELISA).

MTORC1 activation has also been reported in neuronal cultures with primary mtDNA mutation in ATP6, which causes Leigh disease (Zheng et al., 2016). The authors concluded that mTORC1 inhibition had a beneficial effect in cultured neurons since rapamycin increased the ATP content of the cells. Interestingly, mTORC1 activation was also detected in neurons treated with rotenone or oligomycin, which are inhibitors of respiratory chain complexes I and V, respectively (Zheng et al., 2016). In addition, rapamycin extends the lifespan of NDUFS4 knock-out mice (Johnson et al., 2013). These studies indicate that the metabolic stress response we describe in MM might have a role beyond mtDNA maintenance diseases, and our study provides a possible mechanistic explanation for the beneficial effect of rapamycin in mitochondrial dysfunction.

5.3 A COMPLEX NON-CODING LOCUS REGULATES DNA POLYMERASE GAMMA IN THE CENTRAL NERVOUS SYSTEM (III)

To search for putative regulatory elements from the genomic loci of mtDNA maintenance genes, we used 1) experimental genome-wide data from the ENCODE database to find elements that drive wide-spread expression and 2) *de novo* prediction tools to search for regulators that drive highly specific spatio-temporal expression and are not captured with the limited sample set used in ENCODE.

5.3.1 A NON-CODING RNA, *LINC00925,* ASSOCIATES WITH *POLG* EXPRESSION

Promoters, regulatory regions and actively transcribing genes are open chromatin structures and, therefore, sensitive to DNase I treatment. They are thus called DNase I hypersensitive sites (DHS) (Gross and Garrard, 1988; Thurman et al., 2012). Thurman et al. found that the DNase I sensitivity of distal regulatory sites and their target promoters correlate across cell types

since both structures need to be open at the same time in order to have a functional link (Thurman et al., 2012). They performed genome-wide DNase I sensitivity analysis of 125 primary cell and cancer cell lines, correlated DNase I sensitivity of distal DHSs with all promoters in the genome and, thereby, identified putative regulatory sites genome-wide (Thurman et al., 2012). We utilised this DHS data to analyse the genomic regions of the mtDNA maintenance genes *TWNK*, *POLG*, *POLG2*, *SSBP1* and *TFAM* for potential regulatory sites. DHSs were considered putative regulatory sites if they localised within 500 kb from the coding region of the analysed gene and their DNase I sensitivity correlated >0.85 with a DHS in the target promoter.

The DHS analysis revealed 123 putative regulatory sites for *POLG*, which stood out from other analysed genes (*TWNK* (0), *SSBP1* (1) and *TFAM* (5) *POLG2* (0)) (Figure 21). The high number of putative regulatory sites for *POLG* indicated that its transcription is under a complex regulatory circuit. Surprisingly, no DHSs were identified for the POLG accessory subunit, *POLG2,* even though POLG and POLG2 are both required for the functional heterotrimer (Yakubovskaya et al., 2006). This indicates that *POLG2* expression is not rate-limiting for POLG function.

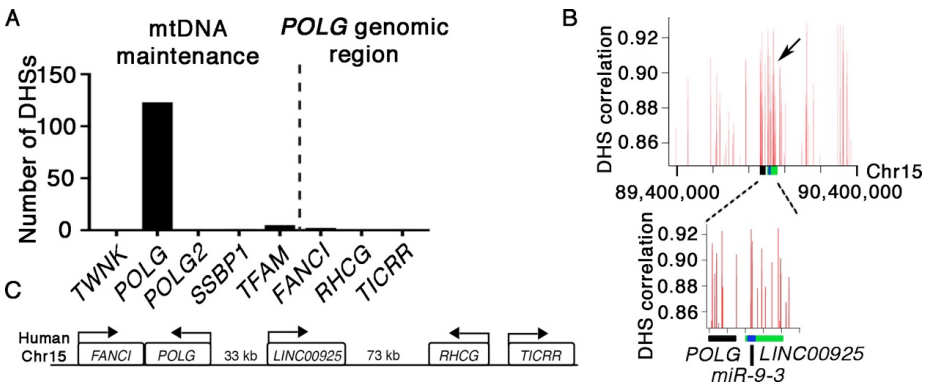

Figure 21 (A) The number of DHSs identified for mtDNA maintenance genes and for the genes in the POLG locus (B) Distribution of *POLG* DHSs (red) in the genomic locus. (C) Schematic picture of genes surrounding *LINC00925*.

The majority of the *POLG* DHSs locates in a cluster that overlaps a lncRNA, *LINC00925*, 33 kb upstream from *POLG* coding region (Figure 21). This implies a regulatory role of *LINC00925* in *POLG* transcription. We detected *LINC00925* expression only in different brain regions and in the SH5Y neuroblastoma line, but no expression was found in muscle or in non-neuronal HepG2 and U2OS cell lines. The mRNA expressions of *Ai854517*, the mouse homolog of human *LINC00925*, and *Polg* significantly correlate during developmental stages of mouse cerebellum (E18.5 and post-natal day 0, 3, 6, 9; 3 mice per time point) (Figure 22). In addition, *in situ* hybridisation of *Polg* and *Ai854517* showed overlapping staining patterns in mouse brain (Figure

22). These results indicate that *LINC00925* is regulating *POLG* expression in the CNS but *LINC00925* knock-down experiment is required to further confirm the functional connection.

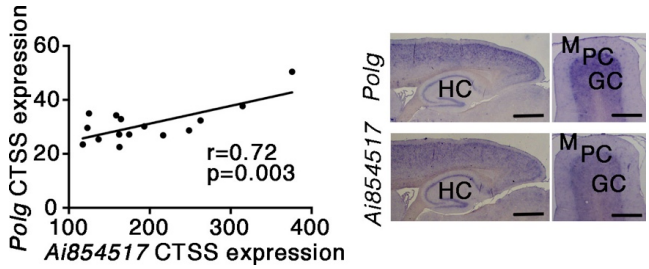

Figure 22 Left, Expression of *Polg* and *Ai854517* in mouse cerebellar development (3 samples per time point: E18, post-natal days 0, 3, 6 and 9). Right, *In situ* hybridisation of *Polg* and *Ai854517* in adult mouse brain. Abbreviations: CTSS, cap analysis of gene expression hits in the transcription start site; HC, hippocampus; M, molecular cell layer; PC, Purkinje cell layer; GC, granule cell layer

5.3.2 *POLG* PROXIMAL PROMOTER DRIVES TISSUE-SPECIFIC EXPRESSION *IN VIVO*

We next tested if the *Polg* proximal promoter has intrinsic tissue-specific properties *in vivo* and used it to drive expression of a marker gene, *lacZ*, in transgenic mouse embryos. The expression of *lacZ* can be detected by an enzymatic staining resulting in precipitation of blue colour in cells. The staining penetrates through the whole embryo at developmental stage E12.5 and we, therefore, used this time point in our studies. To our surprise, *Polg* proximal promoter did not drive ubiquitous transcription, rather *lacZ* expression was detected in specific regions of the nervous system: dorsal root ganglia, developing motoneurons of the neural tube and midbrain. These data indicate that *Polg* proximal promoter is a nervous system specific promoter at E12.5.

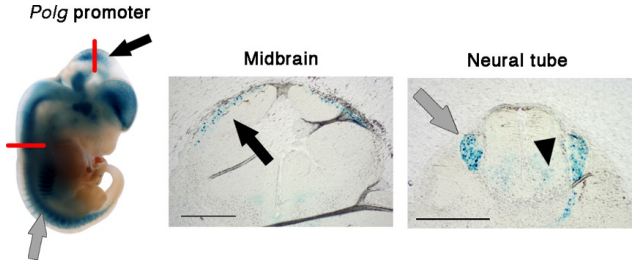

Figure 23 Left, *lacZ* expression driven by *Polg* promoter in E12.5 mouse embryo. Expression in developing midbrain (black arrows) and dorsal root ganglia (grey arrows). Red lines indicate sectioning planes. Scale bars 100 µm.

5.3.3 THREE ENHANCER ELEMENTS ARE PREDICTED TO DRIVE *POLG* EXPRESSION

The CNS-specific properties of the *Polg* proximal promoter guided us to search for enhancer elements (EEs) that might drive *POLG* expression in other tissues. We used Enhancer Element Locator (EEL), which is a *de novo* prediction tool that searches for putative EEs in genomic regions (Hallikas et al., 2006). EEs have clusters of transcription factor binding sites (TFBSs) which form secondary protein-protein interactions with each other. Secondary interactions are critical for the function of EEs since they create a 'protein raft' on the DNA sequence, which mediates the regulatory function of the enhancer. To find EEs from a DNA sequence, EEL searches for regions that have 1) conserved TFBSs and 2) conserved distances between the TFBSs which maintain the secondary interactions (Palin et al., 2006). The analysis provides a score for each predicted EE, which reflects the likelihood of the predicted region to be a functional enhancer. Regions scoring higher than 500 have been shown by others to be good candidates for enhancer regions (Hallikas et al., 2006).

The analysis of the genomic loci of *POLG* and other mtDNA maintenance genes revealed four EEs that scored higher than 500 (Figure 24). These EEs located closer than 100 kb from the analysed genes and were found with human, rat and mouse comparisons. Three of these EEs were predicted to regulate *POLG*. The fourth EE was located 100 kb upstream of *TWNK*, and several genes reside between the putative enhancer and *TWNK* coding region, which indicates that this EE is not a TWNK-specific regulator (Figure 24). It was, therefore, not selected for further analysis. The three *POLG* EEs were all nearly 90% conserved in human, rat and mouse comparisons and the EEL scores for EE1, EE2 and EE3 were 768, 671, 522, respectively. Intriguingly, these enhancers were located in the introns of *LINC00925*, which we earlier linked to *POLG* expression by DHS analysis. Non-coding RNAs are known to function together with EEs, which synergistically induce the transcription of the target promoter (Mattick, 2010).

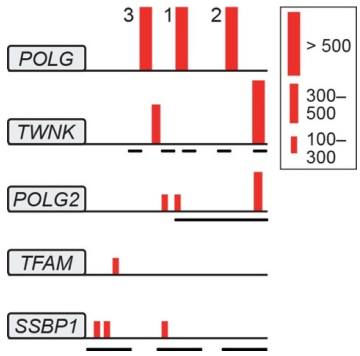

Figure 24 Predictions for enhancer elements in the genomic loci of mtDNA maintenance genes. EEL-scores indicated by red bars. Protein coding genes are indicated by black lines (picture not in scale).

5.3.4 THREE ENHANCER ELEMENTS DRIVE *POLG* EXPRESSION TO SPECIFIC NEURONAL SUBPOPULATIONS IN SPINAL CORD AND BRAIN

We generated reporter mouse lines to validate the enhancer predictions *in vivo*. The vector constructs contained each of the EEs, HSV-TK minimal promoter and *lacZ* as a reporter gene. We chose HSV-TK minimal promoter since it only drives expression in a context of a functional enhancer. All tested enhancers are functional elements *in vivo* and drive expression in distinct regions of the developing nervous system in E12.5 embryos.

The mouse line carrying EE1 showed intense staining in the neural tube, but the most rostral and caudal neural tube regions lacked dorsal expression (Figure 25). EE2 and EE3 showed overlapping expression profiles in the neural tube, and they drive intense expression in the dorsal part, but the expression gradually fades toward the ventral neural tube. In addition, EE2 is active in the dorsal root ganglia (DRG) (Figure 25).

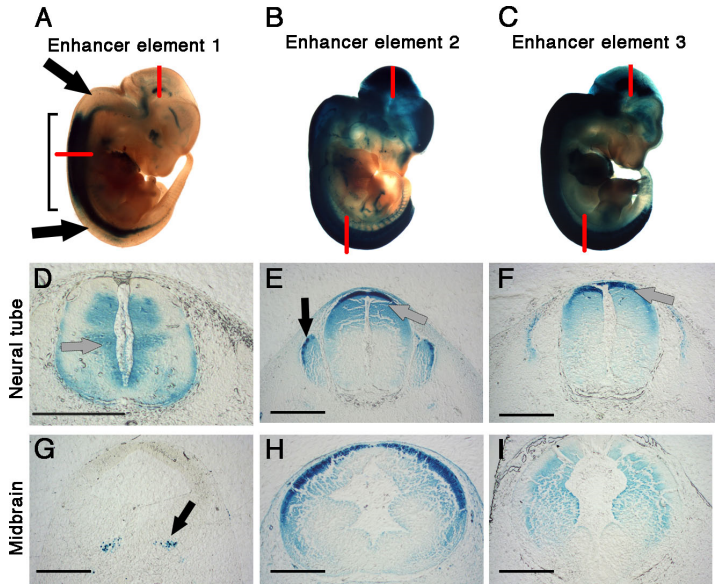

Figure 25 (A)-(C) Expression driven by enhancer elements 1, 2 and 3 in E12.5 mouse embryos. Sectioning planes indicated by red lines. Black line in (A) shows region with dorsal expression in the developing neural tube whereas rostral and caudal regions lack the dorsal expression (black arrows). (D)-(F) Neural tube expression in (D) EE1: proliferating immature neurons (grey arrow), (E) EE2: dorsal neural tube (grey arrow) and dorsal root ganglia (black arrow) and (F) EE3: dorsal neural tube (grey arrow). (G)-(H) Midbrain expression driven by (G) EE1, (H) EE2 and (I) EE3. Scale bars 100 µm.

All three EEs also drive expression in distinct regions of the developing brain. EE1 drives highly specific expression in the midbrain, which co-localises with Islet1/2-positive motoneuron progenitors of the oculomotor complex (Figure 26). These oculomotor neurons innervate the extraocular muscle (EOMs) and

the muscle of the upper eye lid. EE2 is active in the superficial stratum of the superior colliculi, and EE3 drives expression in the dorsolateral midbrain, including both the ventricular and mantle zones (Figure 25).

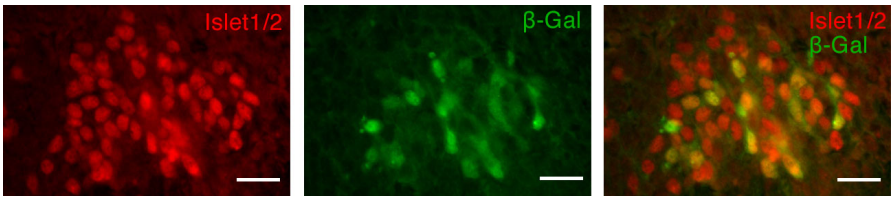

Figure 26 Immunofluorescent co-staining with antibodies against β-Gal and Islet1/2 (marks motoneuron progenitor cells) in EE1 midbrain. The *lacZ* staining of the area is indicated by black arrow in Figure 25G. Scale bars 20 µm.

5.3.5 ENHANCERS DRIVE *POLG* EXPRESSION IN ADULT BRAIN AND SPINAL CORD

Since enhancers have different temporal characteristics, we tested the function of the three POLG EEs in adult mice. EE1 does not drive expression in adult animals, which suggests that its function is restricted to development. However, EE2 and EE3 showed overlapping staining patterns in the adult spinal cord, and they drive expression in the sensory interneurons of the dorsal horns and cells surrounding the central canal (Figure 27). We found that these neurons are often calbindin-positive, which indicates that the enhancers drive expression in glutamatergic interneurons (Figure 28) (Todd, 2010). In addition, EE2 is also active in the adult brain driving strong expression in hippocampus, olfactory bulb and cerebellum (Figure 27). The expression pattern of EE3 in adult brain was inconsistent and, therefore, not conclusive.

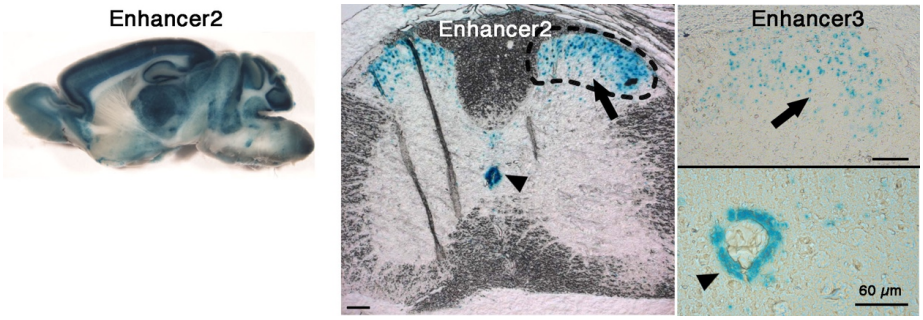

Figure 27 *LacZ* expression in adult mouse brain and spinal cord. Dashed line in the EE2 spinal cord indicates the dorsal horn shown in top panel of EE3 (black arrows). Expression is also detected in cells around the central canal (black arrowheads). Scale bars 100 µm if not stated otherwise.

POLG is the only known mtDNA polymerase and thought to be essential for all cells that have mitochondria. However, our detailed characterisation of the

expression regulation of *POLG* identified regulators that drive the expression only in the nervous system. Other regulators that drive *POLG* expression in other cell types likely exist, but they were not captured by methods used in this thesis. The regulatory locus we identified is, therefore, likely to be part of a complex regulatory circuit that drives the spatio-temporal expression of *POLG*. This is supported by our DHS analysis, which found 123 different regulatory sites for *POLG*, some of which did not overlap with the *LINC00925* coding region. These DHSs are likely *POLG*-regulators driving the expression in other tissues, but the experimental validation of all of them is beyond the scope of this thesis.

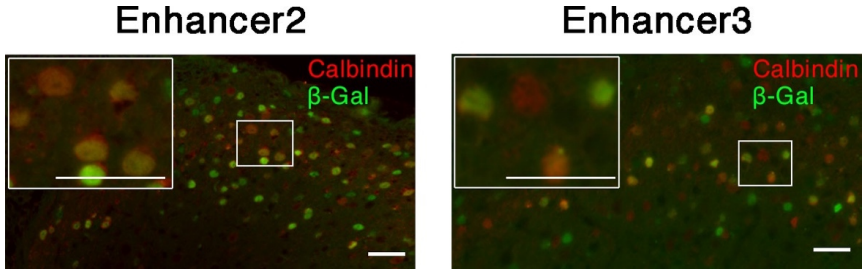

Figure 28 Immunofluorescent co-staining of *lacZ*-expressing neurons in the dorsal horns of the spinal cord. Staining with anti-calbinin (red) and anti-β-Gal (green) antibodies. Scale bars 20 µm.

5.3.6 MIR-9 IS EXPRESSED FROM THE LINC00925-ENHANCER LOCUS

In addition to three EEs, we also found a second regulatory RNA, MIR-9, located within the intron of *LINC00925*. MicroRNAs encoded in the introns of other genes are commonly transcribed together with the primary transcript (Kim and Kim, 2007). Accordingly, MIR-9 and *LINC00925* expressions significantly correlate ($r=0.68$, $p = 0.005$) in mouse cerebellar development, which supports the notion that these two transcripts are co-expressed. Furthermore, the co-expression suggested that the MIR-9 targets are downregulated when *LINC00925* is expressed to drive *POLG* transcription. To identify the targets of MIR-9, we used microRNA target prediction programs, which suggested 6 common targets for MIR-9 (LDLRAP1, MTHFD2, FSTL1, CAPZA1, PRDM1, PRRX). Methylene-tetrahydrofolate dehydrogenase 2, MTHFD2, was especially interesting since it is part of the stress response for mitochondrial replication defects and a previously validated target of MIR-9 (Bao et al., 2016; Nikkanen et al., 2016; Selcuklu et al., 2012).

We suggest that *POLG* and *MTHFD2* are inversely co-regulated in specific neuronal populations, which might have important implications in mtDNA maintenance diseases. The neurons in which the enhancer-lncRNA locus is active also expresses MIR-9, which might block the induction of mitochondrial

stress response during mitochondrial replication disease, which makes these neurons especially vulnerable to *POLG* defects.

5.3.7 NEURONAL DEATH IN THE ENHANCER REGIONS OF A POLG PATIENT

We obtained brain and spinal cord autopsy samples from a patient with POLG-disease and found neuronal death in the enhancer-active regions. The oculomotor nucleus showed spongiotic degeneration, and neurofilament staining of the spinal cord revealed severe loss of neurons in the dorsal columns (Figure 29). This indicates that the enhancer-active regions are especially sensitive to POLG defects, and the death of these neurons might underlie the clinical symptoms of POLG patients: sensory neuropathy and PEO.

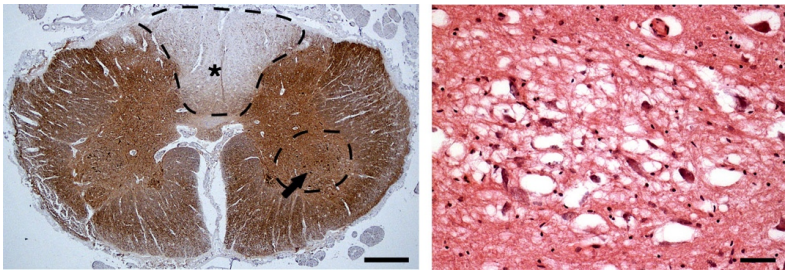

Figure 29 Left, C8-level spinal cord section of a POLG patient (neurofilament staining in brown). Dorsal columns show pallor (star) but motoneurons of the ventral horns are preserved (black arrow). Scale bar 1mm. Right, oculomotor nucleus of POLG patient shows spongiotic degeneration (haematoxylin-eosin staining). Scale bar 50 µm.

5.3.8 IS THE CAUSE OF PEO CENTRAL OR EXTERNAL?

EE1 drives *POLG* expression specifically in the oculomotor complex, which indicates that these neurons have a high requirement for mtDNA replication or repair – possibly due to the high firing rate and the need of mitochondrial respiratory chain function. The oculomotor nucleus innervates the extraocular muscles (EOMs) and the muscle of the upper eyelid, *levator palpebrae superioris*. The dysfunction of these muscles causes PEO, but whether the primary cause is the degeneration of the muscles (external) or the nerve (central), is clinically challenging to determine since the functions of the muscle and the innervating nerve are interdependent. In addition, EOMs accumulate high levels of COX-negative fibres (3.34% in subjects older than 60 years) and mtDNA deletions also during normal aging, which can be misinterpreted as a primary EOM defect in PEO patients (Yu-Wai-Man et al., 2010).

The general MM symptoms highly vary depending on the affected gene. *POLG* MM patients often have severe nervous system involvement with mild

muscle phenotype. The specific expression of *POLG* in the oculomotor complex and the nervous system involvement of *POLG* PEO patients suggest that the cause of PEO in *POLG* cases might be of central origin. In contrast, TWNK patients show clear generalised muscle disease with minor nervous system involvement, and therefore, the cause of PEO in TWNK patients is most likely external. Also, some TWNK patients were found to have accumulation of abnormal mitochondria in EOMs that were severely atrophic, strongly supporting the EOMs to be the cause of PEO (Suomalainen et al., 1992). We, therefore, suggest that PEO can be a primary external or central defect, depending on the affected gene

6 CONCLUSIONS AND FUTURE PROSPECTIVES

This thesis characterised in detail two novel aspects of tissue-specificity in mitochondrial metabolism and dysfunction. First, we identified novel metabolic stress responses activated by mtDNA replication stress and discovered that mTORC1 is the master regulator of these responses. Second, to the best of our knowledge, we are the first to identify tissue-specific regulatory elements of a nuclear-encoded mitochondrial protein, and we describe here a non-coding regulatory locus for *POLG*.

Mitochondrial dysfunction only manifests in specific tissues. Due to the mechanistic advances in the research of mitochondrial metabolism, we know that no simple explanation exists for the tissue-specificity of mitochondrial diseases. The reason why a mitochondrial protein defect only affects a specific tissue, can lie in various signalling or metabolic pathways essential for that cell type. The cell type that is the most dependent on the function of the affected pathway is likely to encounter cellular dysfunction or death, which manifests as mitochondrial disease. The disturbance can be caused by primary or secondary consequences and can be modified by downstream stress responses. These stress responses are possibly, in part, underlying the manifestations of disorders caused by a variety of gene defects. One example of such disorders is PEO, which can be caused by mutations in an array of mitochondrial proteins that do not have obvious functional connection. The gene defects can, however, have similar secondary consequences and stress responses in the affected tissues. Different mutations in the same mitochondrial protein can also lead to completely different tissue manifestations, which indicates that the downstream consequences of defects within one protein can trigger different metabolic or signalling disturbances. Prime examples of such proteins are TWNK and POLG, which were the proteins of interest in this thesis. Our work characterised in detail the metabolic stress responses for mitochondrial diseases revealing novel contributors for the tissue-specific manifestations of mitochondrial diseases.

The detailed characterisation of the biochemical consequences of mitochondrial diseases is valuable when designing specific treatment options. The lack of *in vivo* data from mitochondrial disease models has hindered the development of effective treatments for these diseases. To gain more insight into the disease mechanisms of mitochondrial disorders, we utilised the existing model for MM, the Deletor mouse, and created a novel disease model for IOSCA, which is the first mouse model for mitochondrial encephalohepatopathy. These models are valuable tools for testing metabolic interventions as treatment for mitochondrial diseases.

Our results from the mouse studies identified the metabolic rearrangements caused by mitochondrial replication defects. We found that

mitochondrial myopathy induces a transcription response that is orchestrated by the activation of mTORC1 and is mediated by the ATF4 transcription factor. This leads to 1) remodelling of 1C-metabolism 2) disturbed whole-cellular dNTP pools and 3) diverted glucose consumption into serine biosynthesis and transsulfuration. We show that inhibition of the whole stress response by rapamycin is beneficial for the disease pathogenesis, which indicates that the stress response accelerates disease progression. However, targeting the different branches of the stress response is critical when evaluating the beneficial or detrimental effects of the individual pathways. Some of the activated pathways are likely to be beneficial for the disease, and therefore, specific targeting of the responses that accelerate the disease progression might lead to better treatment strategies.

We also suggest a second new mechanism that might underlie the sensitivity of specific cell populations to mtDNA replication defects. We identified a genomic regulatory locus which drives *POLG* expression in the CNS and functions through three enhancer elements and *LINC00925*. The locus expresses MIR-9 in the same neurons in which it drives POLG expression. Interestingly, these neurons seem to be especially sensitive to POLG defects causing neuronal death in POLG patients in regions where the non-coding locus is active. This raises an intriguing hypothesis that the non-coding locus might sensitise these neurons to mtDNA replication stress. MIR-9 is an attractive candidate to facilitate this effect since it functions *in trans* and targets the mitochondrial folate enzyme MTHFD2. MIR-9, therefore, blocks the induction of the mitochondrial stress response, which can be critical in neurons with mtDNA replication stress. However, these hypotheses need to be tested in appropriate disease models in the future.

In recent years, many new diseases genes have been found due to the development of next-generation sequencing techniques. Despite the rapid identification of new disease genes, many patients suffering from mitochondrial diseases are still without a genetic diagnosis. A part of these patients is likely to have pathogenic mutations in the non-coding regulatory regions which are not captured by exome sequencing. Whole-genome sequencing enables finding these variants but results in a high number of possible pathogenic changes because 1) non-coding regions allow more variation in the DNA sequence than coding-regions, 2) non-coding regions do not have a codon code and 3) prediction tools cannot be used to evaluate the pathogenicity of the variant. Also, the verification of non-coding pathogenic variants often requires *in vivo* experiments due to the lack of cell culture models. Therefore, identifying regulatory regions for mitochondrial disease genes is critical in finding new pathogenic non-coding variants. In this thesis, we identified a regulatory region for the most frequent cause of mitochondrial diseases, *POLG*, which offers the first candidate region for regulatory mutations in mitochondrial disease.

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I want to express my sincere gratitude to all collaborators and co-authors. I especially wish to thank Liliya Euro, Saara Forsström and Christopher Carroll with whom I have experienced the best moments of my scientific career. Liliya, your excitement for science is admirable, and I wish to thank you for the genuine will to help and to teach. Saara, I want to thank you for being an excellent co-worker and a great friend. You make every day at work a joy, and your spirit always lifts me up. Chris, thank you for the scientific discussions and for your friendship.

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